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<b>(54) Title:</b> HUMAN MONOCLONAL ANTI-TUMOR ANTIBODIES  <b>(57) Abstract</b>  Human monoclonal anti-tumor antibodies are isolated from fusion phage single-chain Fv and V <sub>H</sub> antibody libraries constructed from the peripheral blood lymphocytes of immunized cancer patients. Antibodies that bind to tumor cells of the same kind as the patient's are selected, and antibodies that also bind to a human normal cell type are removed. The remaining fusion phage antibodies are cloned and then are tested for binding to at least two normal human cell types. Antibodies that fail to bind to the normal cells are further tested for binding to a panel of tumor cells, typically including the original tumor type and several related and unrelated tumors. Human monoclonal antibodies that bind specifically to the original tumor or also to some other tumors, or that bind to the original tumor and cells from the same developmental lineage, are obtained and sequenced. The selected antibodies can be used to design molecules which are potentially useful for various diagnostic and therapeutic purposes. The single-chain Fv and V <sub>H</sub> libraries from cancer patients are also being used to select antibodies against other targets, such as endothelial cells, which have diagnostic and clinical applications.		

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HUMAN MONOCLONAL ANTI-TUMOR ANTIBODIES**Related Application Data**

This is a continuation-in-part of co-pending U.S. application serial number 08/497,647 filed June 30, 1995.

**Technical Field of the Invention**

5           This invention relates to the preparation of human monoclonal anti-tumor antibodies obtained from immunized cancer patients.

          Among the various strategies for the treatment and prevention of cancer, immunotherapy and vaccination have  
10   long occupied a central position. Although the clinical results have not lived up to early expectations, the remarkable recent advances in our understanding of the immune system and in our ability to manipulate it has refocused interest in cancer immunology. Antibodies and  
15   T-cells are extremely selective agents for targeting specific cell types. Human monoclonal antibodies provide the further advantage of weak immunogenicity in humans.

**Background of the Invention**

          A major focus of cancer immunology is on the iso-  
20   lation of antibodies that react selectively with human tumor cells, since the antibodies could have important applications for targeting diagnostic and therapeutic agents to tumors and for identifying tumorigenic anti-

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gens. The established approach has been to generate large panels of monoclonal antibodies from mice immunized with human tumor cells, and to screen the antibodies for reactivity against the tumor. Despite the enormous effort expended on this approach, few antibodies that react preferentially with human tumors, and none that react specifically with one type of tumor, have been reported.

These results are disappointing but not necessarily conclusive, because the antibodies were generated in an xenogeneic system. Human antigens are generally recognized as foreign by the murine system, and since the tumor antigens are predominately nonspecific, the murine response to human tumors will be correspondingly nonspecific.

Another approach involves generating human monoclonal antibodies from cancer patients by cloning lymphocytes transformed with Epstein-Barr virus or fused with myeloma or B-lymphoblastoid cells. However, the number of clones that can be produced with these procedures is severely limited by technical obstacles, and the antibodies isolated from the clones have shown specificities similar to those obtained with murine monoclonal antibodies.

Further attempts to isolate more specific antibodies will require improved methods of generating and selecting antibodies against human tumors. Two recent developments may be useful in this regard. One involves immunizing cancer patients with autologous tumor cells which have been genetically modified to boost the immunogenicity of the cells (Dranoff, G. & Mulligan, R.C. (1995) *Adv. Immunol.* 58, 417-454). Although there is an extensive normal human repertoire of anti-self antibodies (Griffiths A.D., et al., (1993) *EMBO J* 12, 725-734), indicating that the human immune system can respond to

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self antigens, the humoral response of the immunized cancer patients might be directed preferentially against any non-self antigens expressed by the autologous tumor cells. Numerous such immunization trials are in progress  
5 with melanoma, renal and colon carcinoma, neuroblastoma and breast cancer patients, and others are planned.

The other new development is the introduction of methods for synthesizing virtually the entire repertoire of any person's antibody genes, and for expressing the  
10 encoded antibodies on the surface of a fusion-phage vector (see, for example, Marks, J., et al., (1991) *J. Mol. Biol.* 222, 581-597). The resulting fusion-phage antibody library can be panned to select and clone rare antibodies on the basis of their binding specificities.

15

#### Summary of the Invention

One object of the invention is to provide a new procedure for isolating human monoclonal anti-tumor antibodies from cancer patients.

20 It is a further and more specific object of the invention to provide human monoclonal anti-tumor antibodies that are specific against one or types of tumors, or a specific tumor lineage, and human monoclonal anti-endothelial cell antibodies that can target the tumor vascu-  
25 lature.

These and other objects are achieved by the present invention, which provides a process for synthesizing and isolating human monoclonal antibodies by constructing a fusion phage library from the peripheral blood lympho-  
30 cytes of a cancer patient; selecting for anti-tumor and anti-endothelial antibodies in the phage library in a binding assay with cultured tumor, endothelial or other

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cell types, or with purified antigens; removing extraneous antibodies by absorption against normal human cells; cloning the phage; assaying the specificity of the cloned phage in a binding assay with at least two types of cultured normal cells; and further testing the specificity of cloned phage that do not bind to the cultured normal cells in a further binding assay to cultured tumor cells.

In preferred processes, the cancer patients are first immunized to enhance their immune response against the tumor cells prior to using the peripheral blood lymphocytes for construction of the fusion phage library. In some embodiments, this involves immunization with cultured autologous tumor cells; particularly preferred are immunizations with cultured autologous tumor cells transduced with a cytokine such as  $\gamma$ -interferon, interleukin-4, GM-CSF, or the like.

Fusion phage antibody libraries typically display single-chain Fv antibody fragments. Diverse libraries of immunoglobulin heavy ( $V_H$ ) and light ( $V_L$  and  $V_{\lambda}$ ) chain variable (V) genes are prepared from peripheral blood lymphocytes by polymerase chain (PCR) amplification. Genes encoding single-chain Fv fragments are made by randomly combining heavy and light V-chains using PCR, and the combinatorial library cloned for antibody display on the surface of a phage. *Escherichia coli* phage such as fUSE5 are employed in some embodiments.

In one embodiment, the fusion phage are preferably screened at least two or three times against cultured human tumor cells to enrich for anti-tumor antibodies, and then absorbed against cultured normal human cells to remove extraneous antibodies. The selected phage are then cloned and those that do not bind to at least two normal human cell types are chosen for further characterization in a series of incubations with various types of

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cultured human tumor cells. ELISAs are typically employed as binding assays.

In preferred embodiments, the incubation with tumor cell cultures comprises a series of incubations with at least five different tumor cell cultures, including cultures of the original tumor type, cultures of a related tumor type having the same lineage, and cultures of unrelated tumor types. This procedure identifies antibodies that are specific to the original tumor, to tumors of the same lineage, or to more than one type of tumor. Such antibodies are of use for both diagnostic and therapeutic purposes, and as probes for isolating the cognate tumor antigens.

In one embodiment, single-chain Fv and V<sub>H</sub> antibody fusion phage libraries of melanoma patients immunized with gene-modified autologous melanoma cells are screened for binding to the autologous cells in preferably at least two rounds of selection. Antibodies that bind after both rounds are absorbed against human melanocytes, and the remaining phage are cloned. These phage are first tested by enzyme-linked immunosorbent assay (ELISA) for binding to two normal human cell types such as endothelial cells and fibroblasts. Antibodies that fail to bind to the normal cells are further tested by ELISA for binding to a panel of tumor cells, including several different melanomas and other tumors, and normal melanocytes. Tumor-specific, melanocytic lineage-specific, and melanoma-specific human monoclonal single-chain Fv and V<sub>H</sub> antibodies are identified and sequenced.

The invention further provides single-chain Fv and V<sub>H</sub> antibodies prepared according to the process of the invention, including specific antibodies of defined sequences given hereinafter.

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## Detailed Description of the Invention

This invention is based upon the finding that single-chain Fv and V<sub>H</sub> antibody genes encoding melanoma-specific, tumor-specific, and melanocytic lineage-specific human monoclonal antibodies can be synthesized and  
5 cloned from immunized cancer patients.

In the practice of the invention, fusion phage libraries are constructed from the peripheral blood lymphocytes (PBL) of a cancer patient. By way of illustration and because it is the preferred embodiment, the  
10 patients described in the Examples section are human, but the process is equally applicable to veterinary patients. The patient is preferably immunized to enhance the immune response to the tumor so that a humoral anti-tumor titer  
15 is observed. Any method that enhances the immunologic response to the tumor may be employed, such as genetic manipulation of tumor cells followed by inoculation of the patient with the modified cells, leading to protection against subsequent challenge of wild-type tumor. A  
20 number of such methods have been suggested and are described by Dranoff and Mulligan, cited above, including, but not limited to, systemic administration of recombinant cytokines, tumor antigen-based vaccination schemes, and engineering of tumor cells to express cytokines such  
25 as  $\gamma$ -interferon, GM-CSF, an interleukin such as IL-2, IL-4, and the like, using a retroviral or adeno-associated viral vector. In one embodiment, patients are immunized with gene-modified autologous melanoma cells that have been transfected with human  $\gamma$ -interferon.

30 Virtually the entire repertoire of a person's antibody genes may be synthesized from the peripheral blood lymphocytes (PBL) using polymerase chain reaction (PCR) technology. (See, for example, Winter, G., and Milstein, C., (1991) *Nature* 349, 293-299.) As used here-

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in, "antibody" includes complete antibodies and antibody fragments that bind to antigen such as Fab fragments having non-covalently associated heavy and light chains (and fragments thereof) and single-chain Fv (scFv) antibodies containing heavy and light chains linked by peptides (and fragments thereof). Single-chain Fv antibodies are preferred. The term "anti-tumor antibodies" refers to antibodies to the tumors themselves and tumor-related products and structures, and specifically includes vascular structures supporting tumor growth, proliferation and metastasis. Thus, this invention provides in some instances anti-tumor antibodies to the cancer *per se* and anti-tumor vasculature antibodies.

In the practice of the invention, antibody libraries are typically prepared by first synthesizing cDNAs from poly(A)<sup>+</sup> RNA isolated from the patient's PBL using random hexamers and oligo dT primers supplied by commercially available kits. Genes encoding the variable region heavy chain ( $V_H$ ) for IgG and IgM heavy chains, the  $\kappa$  and  $\lambda$  light chain, and peptide linkers joining the 3'-end of the heavy-chains with the 5' end of the light chain, are synthesized using PCR. The  $V_H$ -linker-light chain ( $V_L$ ) scFv cDNAs are then prepared using PCR. As used herein, the term "cDNA" specifically includes cDNA prepared as outlined above or similar means, and closely related or homologous DNA, particularly DNA that hybridizes under stringent conditions to cDNA so prepared.

The antibody cDNAs so produced are then ligated into the phage display vector such as fUSE5 for *Escherichia coli* described by Scott, J.K. & Smith, G.P. (1990) *Science* 249, 386-390. These typically are filamentous phage that display V-gene libraries on the phage surface. Procedures for the construction of fusion phage antibody

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libraries are given in Marks, et al., cited above, and in the Examples section below.

Fusion phage libraries expressing scFv and V<sub>H</sub> antibodies are screened for binding to the respective tumors in what is described in the literature as a "panning" step. For the practice of the invention, this involves binding of the phage with live cultured tumor cells to select for phage that bind to the cells, and typically involves incubation of the phage with the culture for a time under conditions sufficient to obtain binding of at least a portion of the phage to culture cells. In preferred embodiments, the screen includes at least two or three consecutive pannings of phage against cultures of the same tumor type. Where an autologous tumor cell line was used to immunize a patient, this culture is preferred, but other lines of the same tumor can be substituted.

Preferred procedures for obtaining anti-melanoma antibodies then include extensive absorption against melanocytes to increase the chances of finding antibodies specific to the tumor. For the preparation of other tumor antibodies, the step includes extensive absorption against the normal progenitor cell of the particular tumor if these are available, or alternatively another normal cell type.

Following panning and absorption, the selected phage are then cloned and the specificities of the cloned phage are tested against human normal cultured cells. In preferred embodiments, to enhance the efficacy of this screening step, at least two normal cultured cell lines are employed. Any cell line may be employed in the binding assays; fibroblast and endothelial cells are employed in one embodiment. Any immunoassay that tests for binding specificity familiar to the skilled artisan may be used in this step and subsequent steps involving mea-

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tures of binding with cells, such as, for example radio-immunoassays (RIAs), ELISAs, combinations of these procedures, and the like. Because of their simplicity and ease of use, ELISAs are preferred.

5           Cloned phage that do not bind to normal cells are then tested in binding assays with cultured human tumor cells, including lines of the same tumor type and other types. For anti-melanoma antibodies, for example, preferred panels comprise a series several, e.g., in some  
10       embodiments between five and ten, different human tumor cell cultures comprising autologous melanoma cells, other melanoma cells, unrelated tumor cells, and also normal melanocytes. In especially preferred embodiments, the panel includes at least one cell line from the same lineage as the tumor cell line; in an embodiment illustrating anti-melanoma antibodies in the Examples that follow,  
15       a glioma line derived from glial cells that share a common lineage with melanocytes was employed.

          Any number and mixtures of cultured tumor cell  
20       lines can be used in the panel, and many are readily available from the American Type Culture Collection (A.T.C.C., 12301 Parklawn Drive, Rockville, Maryland 20852) as a stock item. Examples include, but are not limited to, melanoma cells, pancreatic carcinoma cells,  
25       breast carcinoma cells, ovarian carcinoma cells, colorectal carcinoma cells, prostate carcinoma cells, gastric carcinoma cells, renal carcinoma cells, and the like given hereinafter. Examples of cloned phage bearing anti-melanoma antibodies, anti-melanocyte lineage tumor  
30       antibodies, and anti-tumor antibodies, for example, are set out below. The single-chain Fv genes in the selected phage of choice are then sequenced. Examples are set out below in the next section and listed in the Sequence Listing section as SEQ ID NOS 20 to 59.

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Antibody specificity is further assessed using immunohistochemistry of cultured cells and tissue slides so that clones useful for specific diagnostic and therapeutic purposes are identified. Further improvement of the antibodies can be achieved by recombinant DNA procedures involving site-directed mutagenesis and/or variable chain exchanges, followed by panning against the original tumor cells.

10 This invention provides for the cloning and manufacture of human anti-tumor monoclonal antibodies either as fusion phage antibodies or pure antibodies. The cDNAs encoding the antibodies can be employed to express the antibodies in any system known to those skilled in the art, in addition to those set out herein. This invention thus encompasses additional sequences, depending upon vector construction, that facilitate expression of the cDNA in these systems, particularly on a large scale. The invention also encompasses homologous sequences, particularly those having at least about 80% sequence homology.

The anti-tumor antibodies of the invention have at least three important advantages when compared to currently available monoclonal antibodies which are generated in mice. First, since these are human antibodies, the strong immune rejection that occurs when mouse antibodies are injected into humans is avoided. Second, since the antibodies are isolated from fusion phage libraries, their affinity and specificity for a tumor cell line can be improved by genetic manipulations. Third, the antibodies can be readily conjugated to other molecules for diagnostic and therapeutic applications, and for isolating the cognate tumor antigens which could be useful as anti-cancer vaccines.

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Thus, the anti-tumor monoclonal antibodies of the invention are useful in a number of applications. For diagnosis, tagged antibodies of the invention can be used to identify tumor cells in tissue slices from biopsies.

5 For therapy, toxin-linked antibodies of the invention target human tumor tissues (see, for example, the a recent summary by Ghetie, M.-A., and Vitetta, E.S., (1994) *Curr. Opin. Immunol.* 6, 707-714), and such linked antibodies provide immunotoxins for a large variety of  
10 tumors, including melanoma. The effectiveness of scFv and V<sub>H</sub> antibody targeting can also be enhanced using recombinant technology to increase tumor affinity of the antibody. The cytotoxicity of immunotoxins can be tested in vitro against cultured cells, and in vivo using animal  
15 models.

In addition, the invention provides a way to identify antigens that react with scFv and V<sub>H</sub> antibodies by screening cDNA expression libraries with tagged antibodies. Alternatively, antigen can be isolated from a two-  
20 dimensional Western blot and partially sequenced, and a nucleic acid probe encoding that sequence used to isolate a cDNA clone. Antigen may also be isolated by immunoaffinity chromatography of a solubilized labelled melanoma cell preparation. The functions of tumor antigens in  
25 tumorigenesis can be studied to gain additional insight into the development and possible control of cancer.

### Examples

The following examples are presented to further illustrate and explain the present invention and should  
30 not be taken as limiting in any regard.

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## Example 1

This example describes anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells and selection of specific  
5 antibodies from single-chain Fv fusion phage libraries. Some of the research is set out in Cai, X., and Garen, A., *Proc. Natl. Acad. Sci.*, 92: 6537-6541 (1995).

Preparation of peripheral blood lymphocytes (PBL).

PBL were isolated from two melanoma patients (DM341 and  
10 DM414) of Dr. Hilliard Seigler at Duke University Medical Center. The patients had been immunized with cultured autologous tumor cells infected with a retroviral vector carrying the human  $\gamma$ -interferon gene. Each patient received six injections and the sera of both patients  
15 showed a humoral response against the autologous tumor cells as measured by a fluorescence-activated cell sorter (FACS) and solid phase radioimmunoassay (RIA).

After the last injection, 120 ml of blood was removed and the PBL were isolated by centrifugation in  
20 Ficoll Hypaque. The PBL from DM414 were used without fractionation to construct the 414 scFv library. The PBL from DM341 were fractionated by absorption to a culture of autologous melanoma cells growing in a 75 cm<sup>2</sup> flask; after 30 min the unabsorbed PBL were collected and used  
25 to construct the DM341-II scFv library. The melanoma cells were then rinsed twice with culture medium, and the PBL that remained bound to the cells were used to construct the DM341-I scFv library.

The PBL fraction that bound to the melanoma cells  
30 should be enriched for B cells producing membrane-bound IgM, and anti-melanoma antibodies and the other containing the rest of the PBL population should include the mature B cells producing secreted IgG antibodies. As

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described in detail below, the heavy-chain segments of the scFv cDNAs were synthesized from both the IgM and IgG classes of mRNA for the DM341-I and DM414 libraries, and entirely from the IgG class for the DM341-II library.

- 5 Thus the scFv antibodies encoded by each library should correspond mainly to anti-melanoma IgM for DM341-I, entirely to IgG for DM341-II, and to IgM and IgG for DM414.

- Construction of scFv libraries. The protocol  
10 involves four consecutive steps, as follows:  
1) Synthesis of the entire repertoire of first-strand cDNAs from the poly (A)+ RNA of the melanoma patient's PBL. 2) Synthesis by polymerase chain reaction (PCR) of the  $V_H$ - $C_H1$  heavy chain genes for IgG and IgM classes, and  
15 the  $\kappa$  and  $\lambda$  light chains genes, adding complementary coding sequences for a peptide linker at the 3'-end of the heavy chain genes and the 5'-end of the light chain genes. 3) Synthesis by PCR of the  $V_H$ -linker-variable region light chain ( $V_L$ ) scFv cDNAs. 4) Ligation of the  
20 scFv cDNAs into the filamentous phage vector fUSE5, which encodes the complete genome of the phage. Each resulting fusion-phage virion should display 4 or 5 copies of a scFv molecule conjugated to the envelop protein p3.

- Poly (A)+ RNA was isolated from the two PBL sam-  
25 ples, and first-strand cDNAs were synthesized with random hexamers and oligo dT primers, using kits purchased from Invitrogen and BRL. The coding regions for the  $V_H$  -  $C_H1$  heavy chains, and for the  $\kappa$  and  $\lambda$  light chains, were separately amplified by PCR using the following primers,  
30 which are based, in part, on procedures published by Marks, et al., cited above:

A. Constant region forward-primers

IgG: GTCCACCTTG GTGTTGCTGG GCTT (SEQ ID NO 1)

IgM: TGGAAGAGGC ACGTTCTTTT CTTT (SEQ ID NO 2)

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C<sub>x</sub>: AGACTCTCCC CTGTTGAAGC TCTT (SEQ ID NO 3)

C<sub>λ</sub>: TGAAGATTCT GTAGGGGCCA CTGTCTT (SEQ ID NO 4)

B. V<sub>H</sub> back-primers with *Sfi*I site

5 V<sub>H</sub> 1,4,6: ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG  
GTRCAGCTGS WGSAGTCKGG (SEQ ID NO 5)

V<sub>H</sub> 2: ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG  
GTCAACTTAA GGGAGTCTGG (SEQ ID NO 6)

V<sub>H</sub> 3,5: ATGGCTCAGG GTTCGGCCGA CGTGGCCGAG  
GTGCAGCTGK TGSAGTCTGS (SEQ ID NO 7)

10 C. V<sub>κ</sub> back-primers with linker

V<sub>κ</sub> 1,4: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCTGACA TCSWGATGAC CCAGTCTCC  
(SEQ ID NO 8)

15 V<sub>κ</sub> 1,3,6: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCAGAWR TTGTGMTGAC KCAGTCTCC  
(SEQ ID NO 9)

V<sub>κ</sub> 5: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCAGAAA CGACACTCAC GCAGTCTCC  
(SEQ ID NO 10)

20 D. V<sub>λ</sub> back-primers with linker

V<sub>λ</sub> 1,2: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCACAGT CTGYSYTGAC KCAGCCGCC  
(SEQ ID NO 11)

25 V<sub>λ</sub> 3a,3b: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCATCYT MTGWGCTGAC TCAGSMACC  
(SEQ ID NO 12)

V<sub>λ</sub> 4,5: GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC  
GGATCACASG YTRTACTGAC TCAACCGYC  
(SEQ ID NO 13)

30 E. J<sub>H</sub> forward-primer with linker

GCCACCCGAC CCACCACCGC CCGAGCCACC  
GCCACCTGAR GAGACGGTGA CCRKKGTYCC  
(SEQ ID NO 14)

F. J<sub>L</sub> forward-primers with *Sfi*I site

35 J<sub>L</sub> 1: AGTCTAACGT TCGGCCCCAG CGGCCCCACG  
TTTGATYTCC ASCTTGGTCC C (SEQ ID NO 15)

J<sub>L</sub> 2: AGTCTAACGT TCGGCCCCAG CGGCCCCACG

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TTTKATMTCC ASYYKKGTC C (SEQ ID NO 16)  
V<sub>λ</sub>: AGTCTAACGT TCGGCCCCAG CGGCCCCACC  
TARRACGGTS ASCTKGGTC C (SEQ ID NO 17)

5 The sequences are written 5' to 3'. Forward primers are complementary to the sense strand, and back primers are complementary to the antisense strand. Degenerate nucleotides are indicated as follows: Y = C or T; R = A or T; W = A or T; S = C or G; K = T or G; M = A or C.

10 Each PCR mixture contained 2  $\mu$ l from the first-strand cDNA syntheses, 50 pM of a constant region forward-primer, 50 pM of a back-primer, 250  $\mu$ M dNTPs and 2.5 units *Taq* polymerase in buffer as provided (Boehringer Mannheim). A "touchdown" PCR protocol (Don, R.H., et al., (1991) *Nucleic Acids Res.* 19, 4008-4012) was used,  
15 as follows: 3 cycles each of denaturation at 94°C for 1 min, annealing for 2 min, and elongation at 74°C for 3 min; the annealing temperature was varied from 55°C to 46°C in steps of 1°C. The "touchdown" cycles were followed with 10 cycles of annealing at a temperature of  
20 45°C and a 10-min extension at 74°C. The PCR products were purified by electrophoresis in 1% agarose gel and extraction from the gel using the Qiaex kit (Qiagen); the purified DNAs were dissolved in 40  $\mu$ l Tris/EDTA (TE; 10 mM Tris, pH 8.0/1 mM EDTA) buffer.

25 For the next PCR step, the joining-region heavy chain (J<sub>H</sub>) forward-primers listed above were used in combinations with the V<sub>H</sub> back-primers, and the J<sub>L</sub> forward primers were used in combinations with the V<sub>K</sub> and V<sub>λ</sub> primers. The PCR reagents and conditions were the same  
30 as above, except that the primer concentrations were 10 pM and the reaction involved 30 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min followed by extension at 72°C from 10 min. The PCR products were purified as above.

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The complete scFv molecules were synthesized by PCR as follows: 10 ng each of the  $V_H$  and  $V_L$  cDNAs were added to 100  $\mu$ l of PCR reagents and cycled 7 times at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. to form  $V_H$ -linker- $V_L$  molecules. Then 10 pM of the  $V_H$  back primers and 10 pM of the  $J_L$  forward primers were added and cycled again 25 times. The products were precipitated with ethanol and purified by electrophoresis in 1% LMT agarose gel and extraction with  $\beta$ -agarase (New England Biolabs).

The scFv cDNAs and the RF DNA of the *fUSE5* vector were digested with *SfiI* and purified by electrophoresis in 1% LMT agarose gel. Ligation of the cDNA with the vector DNA was done in 100  $\mu$ l reaction mixture containing 1.5  $\mu$ g cDNA, 8  $\mu$ g vector DNA and 2000 units T4 DNA ligase for 16 hours at 16°C.

The ligation products were purified by extraction with phenol/chloroform and precipitation with ethanol, and were dissolved in 20  $\mu$ l of water. The entire purified DNA sample was used to transform DH10B ElectroMax cells (GIBCO BRL), and the cells were plated on 2X TY agar medium supplemented with 12.5  $\mu$ g/ml tetracycline in 24 x 42 cm trays. The total number of transformed cell colonies was about  $4 \times 10^7$  for the DM341-I library,  $5 \times 10^8$  for the DM341-II library and  $2 \times 10^8$  for the DM414 library. The colonies were collected in 2X TY medium supplemented with 12.5  $\mu$ g/ml tetracycline, cultured for 1 hour at 37°C, and the cells were pelleted. The medium was passed through a 0.45 mm filter, and the filtrate, which constituted the scFv library, was stored at 4°C.

Composition of the scFv libraries. Both IgM and IgG  $V_H$  genes were synthesized for the DM341-I and DM414 libraries, and only IgG  $V_H$  genes were synthesized for the DM341-II library. The 341-I library is expected to con-

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tain mainly IgM  $V_H$  genes, since the PBL fraction that bound to DM341 cells was used for the construction of the library. All of the libraries contained  $\lambda$  and  $\kappa$   $V_L$  genes. The fraction of phage with scFv inserts was determined by  
5 PCR synthesis, using primers complementary to phage sequences flanking the insert. The PCR products corresponded to a full-length scFv insert in about 85% of the randomly selected phage clones from each library; in about 10% of the clones the PCR product was truncated.

10       Human cells. a) Endothelial cells were isolated from umbilical cords and cultured; the cells were used after 2 to 6 transfers. b) Melanocytes were isolated from foreskins and cultured; the cells were used after 1 to 4 transfers. c) Melanoma lines DM341 and DM414 correspond to the autologous tumor cell lines used in the  
15 immunization of the patients described above. Other melanoma lines employed were cultures denoted DM 343, ZAZ and SIT1. SIT1-TF2 was a sub-culture of SIT1 which was transduced with the human tissue factor gene. d) Pancreatic carcinoma lines HPAF, Capan-2 and Colo357 were employed. e) The breast carcinoma lines are denoted SK-BR-3 and BT-20. f) The glioma line was Hs683. g) The ovarian carcinoma lines were SK-OV-3 and Hey. h) The colorectal carcinoma lines were HT-29 and Ls180. i) The  
25 prostate carcinoma line was DU145. j) The gastric carcinoma line was MS. k) The renal carcinoma line was Caki-1. Most of the carcinoma lines are available from the A.T.C.C. Culture medium for the melanoma and carcinoma lines was Dulbecco's modified Eagle's medium (DMEM)/10%  
30 fetal calf serum (FCS)/penicillin and streptomycin.

Panning a scFv library. The autologous melanoma lines were grown as an attached monolayer in 24-cm<sup>2</sup> flasks until almost confluent, and after changing the culture medium 3 times, the cells were incubated for 1 hour at  
35 37°C. For the first panning step, the phage from a scFv

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library were precipitated in 4% PEG/0.5 M NaCl and resuspended in water, and about  $10^{11}$  TU were added to the autologous melanoma cells in 2 ml DMEM/10% FCS. The culture flask was shaken gently for 2 hours at room temperature and then the medium was removed and the cells washed rapidly 10 times with phosphate buffered saline (PBS) at room temperature. The phage that remained attached were eluted to the cells in 2.0 ml E-buffer (0.1M glycine pH 2.2/0.1% BSA) for 10 min at room temperature and neutralized with 0.375 ml N-buffer (1M Tris-HCl, pH 9.1). The eluted phage were mixed with 15 ml of log-phase *Escherichia coli* K91 Kan cells, and after 30 min at room temperature, the cells were plated on 2X TY agar/tetracycline at 12.5  $\mu\text{g/ml}$  to amplify the phage. For each subsequent panning step the amplified phage from the previous panning step were precipitated in 4% PEG/0.5M NaCl and resuspended in water, and about  $10^{11}$  phage were used to pan against autologous melanoma cells as described for the first panning step. After overnight incubation at 37°C, the colonies were collected in 2X TY/tetracycline at 12.5  $\mu\text{g/ml}$  medium and cultured for 1 hr at 37°C. The cells were pelleted and the medium was passed through a 0.45-mm filter.

Cloning the phage. After each panning step, the eluted phage before amplification were mixed with *E. coli* K91 Kan cells at low phage-to-cell ratios, and the cells were plated on 2X TY/tetracycline agar and incubated overnight. Individual colonies were inoculated into 2X TY/tetracycline medium and grown overnight with shaking; the cells were pelleted and the medium containing the cloned phage stock was stored at 4°C for ELISA assays.

Absorption against melanocytes. Melanocytes were grown in 35-mm culture dishes until a confluent layer had formed, and the cells were then fixed with glutaraldehyde as described for ELISA reaction and blocked with

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DMEM/FCS. After panning, about 10% of the unamplified phage were added to the melanocytes in DMEM/10% FCS and kept at room temperature for 1 hr with gentle shaking. The unabsorbed phage were then transferred to a fresh culture dish containing fixed melanocytes and the procedure was repeated. After 10 such absorption steps, the unabsorbed were cloned.

ELISA assays. The cells were grown in 96-well plates (Falcon #3072) until almost confluent, washed with PBS and fixed with 0.24% glutaraldehyde for 30 min at room temperature. The fixed cells were washed with PBS and the wells were filled with DMEM/10% FCS and kept for 1 hour at room temperature. The wells were emptied and 200  $\mu$ l of a stock of cloned phage supplemented with 1% BSA was added to each well and kept for 2 hours at room temperature. The wells were washed rapidly 10 times with PBS, and the amount of bound phage was assayed using a peroxidase-conjugated anti-M13 polyclonal antibody and o-phenylenediamine as the peroxidase substrate (Pharmacia Detection Module) following the protocol provided. Absorbance was read at 490 nm. All assays were done in triplicate.

Restriction analysis of scFv cDNAs. The cDNA insert in a phage clone was synthesized by PCR, using the 5'-primer ATTATTATTCGCAATTCCTTTAG (SEQ ID NO 18) and the 3'-primer GAATTTTCTGTATGAGGTTTGTCT (SEQ ID NO 19); these primers hybridize to phage sequences flanking the insert. The purified scFv product was digested separately with AluI for the clones from the DM341 libraries, and with Sau3AI and HinfI for the clones from the DM414 library. The digests were analyzed by electrophoresis in 1.5% agarose gel.

Binding studies. Each library was panned against live cultures of the autologous melanoma cells, and after

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each panning step individual phage randomly selected were cloned and tested by ELISA for binding to the autologous melanoma cells to obtain the following results:

5	Panning step	Clone								
		DM341-I			DM341-II			DM414		
		-	+	++	-	+	++	-	+	++
	0	20	0	0	20	0	0	20	0	0
	1	41	7	0	43	6	0	46	0	1
	2	41	1	6	24	9	14	54	25	719
10	3	20	16	487	45	16	517			

Panning step 0 represents data from the original library. ELISA readings were scored as - for OD < 0.05, + for OD 0.05-0.5, and ++ for OD > 0.5.

15 The data indicate a significant enrichment at each panning step for such clones, with more than 90% showing a strong ELISA reaction after the third step for the DM341 libraries and after the second step for the DM414 library. Some of the phage from the last panning step  
20 were absorbed against cultured melanocytes to remove phage that react with melanocytes.

The phage clones that showed a strong ELISA reaction with the autologous melanoma cells were further tested by ELISA with cultured human endothelial and fibroblast cells, in order to identify clones that do not  
25 react, or react weakly, with two normal cell types, as an indication that the encoded antibodies are at least partly specific for the melanoma cells. The frequency of these clones was 21/517 from the DM341-II library, 18/719  
30 from the DM414 library and 0/487 from the DM341-I library. Thus all of the more specific anti-melanoma antibodies were isolated from the two libraries which contain IgG V<sub>H</sub> genes, and none from the library which contains mainly IgM V<sub>H</sub> genes.

Since some of these antibodies could be identical, having been selected from scFv libraries containing multiple copies of phage with the same scFv cDNA insert, the insert in each clone was first analyzed by restriction mapping to identify inserts with different restriction patterns which should encode different antibodies. One representative clone for each of the different antibodies, seven from the DM341-II library and six from the DM414 library, was chosen for further ELISA tests against a panel of human tumor lines. The panel contained melanoma, glioma, pancreatic, breast, ovarian, colon, prostate and gastric tumor lines, and also cultured normal melanocyte, endothelial and fibroblast cells (Table 1).

Table 1. ELISA assays of selected anti-melanoma scFv fusion-phage clones for binding to cultured tumor lines and normal cells.

ELISA assay, relative binding unit																			
Clone	Melanoma					Endo- thelial	Fibro- blast	Melan- ocyte	Glioma	Ovarian		Breast		Pancreatic			Colorectal		Gastric
	1	2	3	4	5					1	2	1	2	1	2	3	1	2	
84	1.81	1.71	1.79	1.74	1.88	1.35	1.85	1.71	1.21	1.61	1.69	1.81	1.66	1.62	1.91	1.79	1.83	1.83	1.80
D5	1.16	0.42	0.50	0.68		0.06	0.01	0.21	0.35	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.01	0.00	0.00
D33	1.15	0.54	0.47	0.67		0.06	0.02	0.32	0.58	0.04	0.03	0.08	0.01	0.00	0.06	0.07	0.06	0.13	0.09
Z78	0.75	0.16	0.26	0.43		0.05	0.05	0.20	0.36	0.01	0.01	0.04	0.00	0.00	0.01	0.00	0.02	0.02	0.01
G57	1.20	0.52	0.58	0.79		0.08	0.01	0.36	0.40	0.01	0.00	0.05	0.00	0.00	0.00	0.00	0.01	0.01	0.00
H18	1.17	0.36	0.45	0.66		0.05	0.02	0.26	0.45	0.02	0.00	0.07	0.00	0.00	0.01	0.00	0.02	0.00	0.00
K2	0.58	0.20	0.30	0.43		0.00	0.01	0.20	0.21	0.02	0.01	0.05	0.00	0.00	0.01	0.01	0.03	0.04	0.01
K10	0.56	0.24	0.52	0.18		0.03	0.05	0.14	0.65	0.11	0.16	0.27	0.12	0.15	0.17	0.21	0.22	0.26	0.18
V13	1.04	1.51	0.73	1.59	1.11	0.00	0.04	0.04	0.90	0.02	1.61	0.03	0.31	0.46	0.86	0.20	0.00	0.07	0.02
V73	0.04	0.14	0.02	0.69	0.30	0.00	0.01	0.03	0.84	0.02	1.58	0.07	0.09	0.05	0.04	1.16	0.00	0.00	0.59
V86	0.87	0.92	0.38	0.74	0.59	0.00	0.02	0.03	0.01	0.00	0.00	0.01	0.00	0.01	0.21	0.01	0.00	0.00	0.00
V373	0.58	0.65	0.98	0.87	0.80	0.07	0.05	0.46	0.53	0.50	0.14	0.50	0.41	1.02	0.25	0.57	0.95	0.76	1.67
V474	0.40	1.76	1.04	1.60	1.71	0.00	0.05	0.00	0.32	0.01	0.73	0.00	0.20	0.24	0.32	0.11	0.04	0.03	0.03
V575	0.77	0.88	0.87	0.26	0.59	0.06	0.00	0.15	1.57	0.07	0.57	0.00	0.01	0.00	0.31	0.00	0.06	0.00	0.08

For each clone an equal aliquot from the same stock was used in all assays. Although stocks for all clones were prepared with the same procedure, phage titers could differ. Therefore, ELISA assays for each clone can be compared to obtain an estimate of the relative binding specificities of the clone for different cells. However, comparison of ELISA assays among different clones might not be significant. Clone 84 is a non-

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specific anti-melanoma control isolated from the panned DM341-II library. Clones D, G, H, K, and Z were isolated from the DM341-II library after the third panning step; D and Z clones were isolated before, and the others after, absorption to melanocytes. V clones were isolated from the DM414 library after the second panning step and absorption to melanocytes. ELISA values are the averages of three assays that generally agreed within 10%. Numbered cell lines are as follows: (i) melanoma—1, DM341; 2, DM343; 3, SIT1-TF2; 4, ZAZ; 5, DM414, (ii) ovarian—1, SK-OV-3; 2, Hey, (iii) breast—1, SK-BR-3; 2, BT-20, (iv) pancreatic—1, HpaF; 2, Colo 347; 3, Capan-2, (v) Colo-rectal—1, HT-29; 2, Ls180. Other lines are identified above. Each ELISA plate included two wells used as a baseline, one containing cells only and another containing cells and a scFv clone, isolated from the original library, which did not show significant binding to any of the cell lines. The average of the ELISA readings of the two wells, which was generally <0.1, was subtracted from the readings of the other wells in the plate. All assays were done in triplicate, and agreement was generally within 10%.

Six of the clones from the DM341-II library (D5, D33, Z78, G57, H18, and K2) reacted strongly only with melanoma, melanocyte and glioma cells. Since these three cell types share a common developmental lineage from embryonic neural crest tissue, the six reactive antibodies appear to be specific for that lineage. The remaining clone K10 reacted also with the other tumor lines which have different lineages.

Four of the clones from the DM414 library (V13, V73, V86 and V474), in contrast to the clones from the DM341-II library, did not react, or reacted weakly, with melanocytes. Clone V86 shows the tightest association with melanoma, reacting strongly only with the melanoma

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lines and one pancreatic tumor line. Clones V13, V73 and V474 reacted with melanoma lines and also with several other tumor lines. The remaining clones V373 and V575 reacted with melanocytes as well as melanomas and other tumor lines, similar to clone K10 from the DM341-II library.

The clone binding with the panel of 21 human tumor lines, comprising melanomas and 8 other types of tumors, and also normal melanocyte, endothelial and fibroblast cells thus identifies three classes of anti-melanoma antibodies: One is a melanoma-specific class which shows a strong ELISA reaction almost exclusively with melanoma lines; the second is a tumor-specific class which shows a strong ELISA reaction with melanoma lines and several of the other tumor lines but not with normal melanocyte, endothelial or fibroblast cells; the third is a lineage-specific class which shows a strong ELISA reaction with the melanoma lines and melanocytes, and also with a glioma line which shares a common melanocytic lineage, but not with other tumor lines or with normal endothelial and fibroblast cells. Most of the antibodies reacted with all of the melanoma lines tested, and therefore appear to recognize antigens common to all human melanomas. The ELISA results for clone V86 were confirmed by immunohistochemistry, reacting biotin-labelled fusion phage with cell lines and staining with the ABC/DAB kit from Vector Laboratories.

Clone sequencing. Ten of the clones listed in Table 1 were sequenced. The complete sequence of the single-chain Fv cDNA insert in the fusion phage was determined for each clone. The sequences are set out in the Sequence Listings section as SEQ ID NOs 20 to 38. The sequences for the Complementary Determining Regions (CDR) for each clone are shown in Table 2 below. Note that V86 and V575 do not contain a complete light chain.

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Table 2. Amino acid sequences of the Complementary Determining Regions CDR1, CDR2 and CDR3 of selected cloned antibodies. These sequences are taken from the complete single-chain Fv sequences for each clone.

5	A. <u>Heavy Chains</u>			
	Clone #	CDR1	CDR2	CDR3
10	H18	NHWFH	WVSPNTGATKYAEMFQG	AAGSSYYFGMDI
	G57	DYWMS	AISGSGGSTYYTDSVKG	EMNYFSHAMDV
	D33	NHWFH	WVSPNTGATKYAEMFQG	AAGSSYYFGMDI
	Z78	GHAMH	AISGNGGSTYYSDSVKG	DWYPDSWSGYAVDGLDV
	V13	SYAMS	AISGSGGSTYYADSVKG	GVAPFDY
	V73	SYWIG	IIYPGDS DTRYRPSFQG	LTPDDYGGNTPDY
	V86	SYAMS	AISGSGGSTYYADSVKG	GWGLRGEEDYMDV
	V373	SSYIH	VINPSGGNTIYARNFQG	DRRYCSGGSCYAEVVY
	V474	SYTMS	AISGSGGSTYYADSVKG	GVAPFDY
	V575	SYAMH	VISYDGSNKYYADSVKG	GFPYGGNSDYGMDV
25	B. <u>Light Chains</u>			
	Clone #	CDR1	CDR2	CDR3
20	H18	SGSSSNIGSNYVY	RNNQRPS	QSYDNSLNGYV
	G57	SGSSSNIGSNYVY	RNNQRPS	ASWDDSLRGYV
	D33	RSSQTLVFSDGHNYVN	ELSNRDP	IH/QGTLCPFT
	Z78	SGSSSNIGNNAVN	YDDLPS	AAWDDSLNGP
	V13	RASQSISSYLN	KASSLES	QQYNSYSRT
	V73	SGSSSNIGNNYVS	ENNKRPS	GTWDSSLSAEV
	V86	not present	not present	not present
	V373	SGSSSNIGNNYVS	ENNKRPS	QSYDSSLSGYV
25	V474	RASQSISSYLN	KASSLES	QQYNSSSRT
	V575	not present	not present	not present

The table uses the following abbreviations for the amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val, W, Trp; and Y, Tyr. Full amino acid sequences using the U.S. Patent Office format are set forth hereinafter in the Sequence

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Listing section of this application. Numbering corresponds as follows: In part A above, the CDR1, CDR2, and CDR3 fragments of the antibody heavy chains are respectively denoted residues 31 to 35, 50 to 66, and 99 to 110 of SEQ ID NO 20 for clone H18; residues 31 to 35, 50 to 66, and 98 to 108 of SEQ ID NO 22 for clone G57; residues 30 to 34, 49 to 65, and 98 to 109 of SEQ ID NO 24 for clone D33; residues 31 to 35, 50 to 66, and 89 to 105 of SEQ ID NO 26 for clone Z78; residues 31 to 35, 50 to 66, and 112 to 121 of SEQ ID NO 28 for clone V13; residues 31 to 35, 50 to 66, and 99 to 111 of SEQ ID NO 30 for clone V73H; residues 31 to 35, 50 to 66, and 99 to 118 of SEQ ID NO 32 for clone V86; residues 29 to 33, 47 to 63, and 96 to 111 of SEQ ID NO 34 for clone V373H; residues 31 to 35, 50 to 66, and 99 to 105 of SEQ ID NO 36 for clone V474; and residues 31 to 35, 50 to 66, and 99 to 112 of SEQ ID NO 38 for clone V575.

For part B above, the CDR1, CDR2, and CDR3 fragments of the antibody light chains are respectively denoted residues 23 to 35, 51 to 57, and 90 to 100 of SEQ ID NO 21 for clone H18L; residues 23 to 35, 51 to 57, and 90 to 100 of SEQ ID NO 23 for clone G57; residues 24 to 39, 55 to 61, and 94 to 102 of SEQ ID NO 25 for clone D33; residues 23 to 35, 51 to 57, and 88 to 97 of SEQ ID NO 27 for clone Z78; residues 24 to 34, 50 to 56, and 89 to 97 of SEQ ID NO 29 for V13; residues 24 to 36, 52 to 58, and 91 to 101 of SEQ ID NO 31 for V73; residues 23 to 35, 51 to 57, and 90 to 100 of SEQ ID NO 35 for V373; and residues 24 to 34, 50 to 60, and 93 to 101 for V474.

Note that since the lineage-specific class of anti-melanoma antibodies is an anti-self class, some or all of those antibodies might also be part of the normal anti-self repertoire of unimmunized persons. However, unlike the normal human anti-self repertoire which is composed mainly of IgM antibodies, the lineage-specific

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anti-melanoma antibodies are isolated from the 341-II library which contains only IgG V<sub>H</sub> genes, but not from the 341-I library which contains mainly IgM V<sub>H</sub> genes. IgG antibodies are generated in response to an antigenic stimulus, which for the melanoma patients in this study was the immunization with autologous tumor cells. Since there was a humoral response to the autologous tumor exhibited by the patients, the lineage-specific anti-melanoma antibodies appear to have been induced by the immunization. This conclusion is supported by the sequence analyses of the scFv genes for four of the lineage-specific antibodies, which show numerous differences in the CDR regions from the nearest germline V-genes.

The sequences show that, although the two immunized melanoma patients were treated with the same immunization protocol, different classes of anti-tumor antibodies are produced. Thus, the lineage-specific class was isolated from the 341-II library and the melanoma-specific class from the 414 library.

The sequences also show the variety of antibodies generated by the procedure, and indicate the enormous size of the untapped pool of antibodies that can be accessed from blood samples to expand the repertoire of human anti-tumor antibodies.

## Example 2

A melanoma-specific V<sub>H</sub> antibody cloned from a fusion phage library of a vaccinated melanoma patient described in Example 1 was further investigated. Some of the research is set out in Cai, X., and Garen, A., Proc. Natl. Acad. Sci. 93: 6280-6285 (1996).

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As the previously described ELISA tests for binding of the V86 fusion phage to a panel of human metastatic melanoma and carcinoma cell lines and primary cultures of normal melanocytes, endothelial and fibroblast cells in Example 1 showed, measurable binding occurred only to the melanoma cells. The strict specificity of V86 for melanoma cells was confirmed by immunohistochemical staining tests with cultured cells and frozen tissue sections in this Example: The V86 fusion phage stained melanoma cell lines, but did not stain carcinoma cell lines or cultured normal cells; V86 also stained specifically the melanoma cells in sections of metastatic tissue but did not stain any of the cells in sections from normal skin, lung and kidney or from metastatic colon and ovarian carcinomas and a benign nevus.

An unexpected finding was that V86 contained a complete  $V_H$  domain but only a short segment of a  $V_L$  domain which terminated before the CDR1 region. This  $V_L$  deletion resulted from the occurrence in the VL cDNA of a restriction site which was cleaved during construction of the scFv library. Thus V86 is essentially a VH antibody. The effect of adding a  $V_L$  domain to V86 was examined by constructing scFv fusion phage libraries in which V86 was coupled to  $V\lambda$  or  $V\kappa$  domains from the original scFv library of the melanoma patient, and then panning the libraries against melanoma cells to enrich for the highest-affinity antibody clones. None of the V86 -  $V\lambda$  clones showed significant binding to melanoma cells in ELISA tests; although binding occurred with most of the V86 -  $V\kappa$  clones it was generally weaker than the binding of V86. These results indicate that most of the  $V_L$  domains in the original scFv library reduce or eliminate the affinity of V86 for melanoma cells. V86 was further characterized by immunohistochemistry with cultured cells and tissue sections.

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*Human cells.* Cell lines and isolates were as described in Example 1 above, except that melanoma line A2058 obtained from the A.T.C.C. was also employed.

*Immunoperoxidase staining of cells and tissue sections.* The cultured cells were grown in 16-well culture chambers (Lab-Tek Chamber Slide) until about 50% confluent, washed with PBS and fixed with 0.24% glutaraldehyde for 30 min at room temperature. The fixed cells were washed with PBS and the wells were filled with 2% FCS in PBS and kept for 1 hr at room temperature. The wells were emptied and 200  $\mu$ l of a stock of cloned V86 fusion phage or fUSE5 control phage diluted 1:1 with 2% FCS in PBS was added to each well and kept for 2 hr at room temperature, and the wells were washed 3 times with PBS for 5 min each. The bound phage were detected by reacting first with a peroxidase-conjugated anti-M13 polyclonal antibody (Pharmacia) diluted 1:200 and then staining with a diaminobenzidine+H<sub>2</sub>O<sub>2</sub> substrate (Vector Labs). Tissue sections cut from frozen samples of melanoma tumors or normal skin were attached to glass slides and fixed in 0.24% glutaraldehyde at room temperature for 10 min. The fixed sections were used for immunohistochemistry as described for cultured cells.

*DNA sequencing.* A single bacterium colony carrying a phage clone was inoculated into 50 ml 2xTY tet broth and grown overnight; the bacteria were removed by centrifugation and the supernatant medium was filtered through a 0.45  $\mu$ m membrane. The phage in the medium were precipitated by adding 1/10 volume of a 20% polyethylene glycol 8000 + 2.5 M NaCl stock solution, and the phage were pelleted and resuspended in PBS. The precipitation procedure was repeated once and the phage pellet was resuspended in 100  $\mu$ l water. The phage DNA was extracted twice with phenol+chloroform and once with chloroform, and the DNA was precipitated with ethanol+NaAc and resus-

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pended in 50  $\mu$ l water. The primer for sequencing the VL domain was TGATTTTCTGTATGAGG (SEQ ID NO. 39) which hybridizes 90 bases away from the 5'-end of gene-3 of the phage; the primer for sequencing the VH domain was ACCCGACCCACCAACCGCCCGA (SEQ ID NO. 40) which hybridizes to the linker of the scFv molecule.

*Construction of V86-V $\lambda$  and V86-V $\kappa$  libraries.* The cDNA encoding the VH domain of V86 was synthesized by PCR from the V86 fusion phage clone as described in Example 1. The sequence of the 5'-primer was ATTATTATTCGCAATTCC-TTTAG (SEQ ID NO. 18) which hybridizes to the gene-3 of the phage, and the sequence of the 3'-primer was GCCACCCGACCCACCAACCGCCCGAGCCACCTGARGAGACGGTGACCRKKGTYCC (SEQ ID NO. 14) which includes part of the J $\mu$  and linker sequences. The cDNAs encoding the V $\kappa$  and V $\lambda$  domains were synthesized from PBL of melanoma patient DM414 as described above. To construct the V86-V $\kappa$  and V86-V $\lambda$  scFv cDNAs the complementary linker sequences were hybridized and the cDNAs were synthesized by PCR. The procedure involved adding 10 ng of V86 cDNA and 10 ng of V $\kappa$  or V $\lambda$  cDNAs to 100  $\mu$ l of PCR reagents and cycling seven times at 94° C for 1 min, 60° C for 1 min, and 72° C for 2 min; 10 pM of a mixture of VH back-primers and 10 pM of J $\kappa$  or J $\lambda$  forward primers were then added and the PCR synthesis was continued for 25 cycles. The PCR products were precipitated with ethanol and purified by electrophoresis in 1% LMT agarose gel and extraction with  $\beta$ -agarase. To construct the fusion phage libraries, the scFv cDNAs and the replicative form of the *fUSE5* DNA were digested with SfiI and ligated in 100  $\mu$ l of reaction mixture containing 1.5  $\mu$ g of scFv cDNA, 8  $\mu$ g of vector DNA and 2000 units of T4 DNA ligase for 16 hrs at 16° C. The ligation products were purified by extraction with phenol+chloroform and precipitation with ethanol, and the purified DNA was used to transform DH10B ElectroMax cells (GIBCO/BRL). The cells were plated on 2xTY tet agar. The total number of

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transformed clones was  $4 \times 10^7$  for the V86-V $\kappa$  library and  $1 \times 10^8$  for the V86-V $\lambda$  library. The fusion phage stocks for each library were prepared from the transformed cells. The fraction of phage with scFv inserts, as determined by PCR synthesis using primers complementary to phage sequences flanking the insert, was 75% (15/20) for the V86-V $\kappa$  library and 83% (19/23) for the V86-V $\lambda$  library.

*Panning the V86-V $\kappa$  and V86-V $\lambda$  libraries.* The melanoma cell line A2058 was grown as an attached monolayer in 24 cm<sup>2</sup> flasks until almost confluent, washed with PBS and fixed with 0.24% glutaraldehyde for 10 min at room temperature. The fixed cells were washed with PBS and blocked with DMEM+10% FCS for 1 hr at room temperature. The phage from the V86-V $\kappa$  and V86-V $\lambda$  libraries were precipitated in 4% PEG+0.5 M NaCl and resuspended in water, and about  $10^{11}$  phage in 2 ml DMEM+10% FCS were added to the melanoma cells. The culture flask was shaken gently for 2 hr at room temperature and the medium was removed and the cells washed rapidly 10 times with PBS at room temperature. The attached phage were eluted from the cells in 2 ml of E-buffer for 10 min at room temperature and immediately neutralized with 0.375 ml N-buffer (2). The eluted phage were mixed with 15 ml of *E. coli* K91 Kan cells and after 30 min at room temperature the cells were plated on 2xTY tet agar. The colonies were collected in 50 ml 2xTY tet medium and incubated for 1 hr. The bacteria were pelleted and the supernatant medium was filtered through a 0.45  $\mu$ m membrane. For each subsequent panning step the amplified phage from the previous panning step were precipitated in 4% PEG+0.5 M NaCl and resuspended in water, and about  $10^{11}$  TU of the phage were used for panning against melanoma cells A2058 as described for the first panning step.

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*Preparation of fusion phage clones.* The phage were mixed with *E. coli* K91 Kan cells at low phage to cell ratios and the cells were plated on 2xTY tet agar. Individual colonies were inoculated into 2 ml 2xTY tet medium and grown overnight. The cells were pelleted and the medium containing the cloned phage was used for the initial ELISA assays. The medium was filtered through a 0.45  $\mu$ m membrane and precipitated in 4% PEG+0.5 M NaCl twice. The phage titer in each cloned stock was determined and adjusted to contain  $10^{10}$  phage transforming units (TU) per ml.

*ELISA assays.* The cells were grown in 96-well microtiter plates until almost confluent, washed with PBS and fixed with 0.24% glutaraldehyde for 30 min at room temperature. The fixed cells were washed with PBS, and the wells were filled with DMEM+10% FCS and kept for 1 hr at room temperature. The wells were emptied and 100  $\mu$ l of a stock of cloned phage diluted 1:1 with DMEM+10% FCS was added to each well. After 2 hr at room temperature the wells were washed rapidly 10 times with PBS and the amount of bound phage was assayed using a peroxidase-conjugated anti-M13 polyclonal antibody and 0-phenylenediamine as the peroxidase substrate (Pharmacia Detection Module). All assays were done in triplicate.

*Restriction analysis of V86-Vk and V86-V $\lambda$  cDNAs.* The cDNA insert in a phage clone was synthesized by PCR using the 5'-primer ATTATTATTCGCAATTCCTTTAG (SEQ ID NO. 18) and the 3'-primer GAATTTTCTGTATGAGGTTTTGCT (SEQ ID NO. 19) which hybridize to phage sequences flanking the insert. The purified cDNA products were digested separately with *Sau*3AI and *Hinf*I, and the digests were analyzed by electrophoresis in 2% agarose gel.

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Results. The antibody V86 was selected from a scFv fusion phage library of a melanoma patient by panning against the autologous tumor cells. Previous ELISA tests with a panel of melanoma lines, carcinoma lines and primary cultures of normal cell types including melanocytes showed that V86 fusion phage could bind to the melanoma cells but not to the carcinoma cells or normal cells (Example 1). The melanoma-specific binding of V86 was further tested by immunohistochemistry with several of the tumor cell lines and normal cells used for the ELISA tests. V86 showed a staining reaction with the melanoma cells but not with either the carcinoma cells or normal cells, confirming the ELISA results. Because the reaction of V86 with melanoma cells might involve an antigen expressed by the cultured cells but not by melanoma tumors *in vivo*, additional immunohistochemistry was done with sections of frozen human metastatic melanoma tissue and normal skin tissue. V86 showed a staining reaction with the melanoma tissue but not with the connective tissue in the melanoma sections or with any of the tissues in the section of normal skin; V86 also failed to stain any of the tissues in sections of frozen normal lung and kidney or of metastatic colon and ovarian carcinomas and a benign nevus. These results demonstrate that V86 can bind specifically to melanoma cells in a metastatic tumor as well as to cultured melanoma cells. The staining of the melanoma sections appears to concentrate along the borders of the melanoma cells, suggesting that the antibody binds at the cell surface.

The sequence of the V86 antibody, as deduced from the sequence of the cDNA insert in the fusion phage, shows a complete  $V_H$  domain followed by the linker which couples the  $V_H$  and  $V_L$  domains in a scFv molecule; however the  $V_L$  domain terminates before the CDR1 region (Table 3).

Table 3. Amino Acid Sequence of Clone V86.

FR1	CDR1	FR2	CDR2
QVQLVQSGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVA	AISSGGSTYYADSVKG
FR3	CDR3	FR4	
RFTISRDN SKNTLYLQMNSLRAEDTAVYYCAR	GWGLRGEEGDYYMDV	WGKGTIMVTSS	
LINKER	FR1		
GGGSGGGSGGGGS	SYELTQEPRGGGTQLTVLGGAAGA		

The amino acid sequence was derived from the nucleotide sequence of the cDNA insert in the V86 fusion phage (SEQ ID NOs 32 and 33; GenBank data base accession no.

- 5 U58144). The complete  $V_H$  domain is followed by the linker and the truncated  $V_L$  domain.

- The reason for the premature termination of the  $V_L$  domain is the presence of an *SfiI* restriction site which was cut in the process of cloning the V86 scFv cDNA into the fusion phage, resulting in the deletion of the distal  $V_L$  segment.
- 10

- The next series of experiments was designed to test the effect of adding a  $V_L$  domain to V86 on the specificity and affinity of the antibody for melanoma cells.
- 15 Two scFv fusion phage libraries were constructed for this purpose, one expressing V86- $V_K$  antibodies and the other V86- $V_\lambda$  antibodies. The  $V_K$  and  $V_\lambda$  domains for these libraries were derived from the repertoire of  $V_L$  domains in the original scFv library used for the isolation of V86.
- 20 Individual clones were isolated from each library, and those containing different  $V_\lambda$  or  $V_K$  domains, as determined by restriction mapping the cDNAs in the fusion phage, were tested by ELISA for binding to the melanoma cell line A2058 (Table 4).

Table 4. Effect of Different V $\lambda$  and V $\kappa$  Domains on the Binding of V86 to Melanoma Cells.

clones	ELISA absorbance			
	+++	++	+	-
V86- V $\lambda$	0	0	0	50
V86- V $\kappa$	2	20	28	18

The V86- V $\lambda$  and V86-V $\kappa$  clones are random isolates from the two fusion phage libraries, each expressing the V86 V $_H$  domain linked either to V $\lambda$  or V $\kappa$  domains from the original scFv library. The melanoma cell line for the ELISA tests was A2058. The ELISA absorbance scales are as follows: +++ >0.8; ++ 0.8-0.5; + 0.5-0.1; - <0.1. The absorbance for V86 without a V $_L$  domain is 1.0.

None of the V86-V $\lambda$  clones showed detectable binding, indicating that V $\lambda$  domains are incompatible partners for V86; most of the V86-V $\kappa$  clones showed significant binding but it was generally weaker than the binding of V86. Thus most of the V $_L$  partners for V86 reduce or eliminate its capacity to bind to melanoma cells. The V86-V $\lambda$  and V86-V $\kappa$  libraries were then panned twice against melanoma cells to enrich for any higher affinity antibodies which might be present, and clones of the panned phage containing different V $\lambda$  or V $\kappa$  domains were tested by ELISA for binding to the melanoma line A2058. As before, binding occurred with the V86-V $\kappa$  clones and not with the V86-V $\lambda$  clones. The relative binding affinities for melanoma cells of 15 panned V86-V $\kappa$  clones were compared with V86, using a Scatchard plot assay to determine K $_d$  values for binding to a melanoma cell line. The relative binding affinity was highest for V86, indicating that V86 functions more effectively as a V $_H$  than as a scFv anti-melanoma antibody. The panned V86-V $\kappa$  clones

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were also tested by ELISA for specific binding to melanoma cells, using a panel of cells consisting of the melanoma line A2058, 4 carcinoma lines and primary cultures of melanocyte, endothelial and fibroblast cells. All of  
5 the V86-V $\kappa$  clones bound only to the melanoma cells, indicating that addition of a V $\kappa$  domain to V86 affects only its affinity but not its specificity for melanoma cells.

*Discussion.* The strict specificity of the human antibody V86 for melanoma cells first demonstrated by  
10 ELISA tests with a panel of human melanoma and carcinoma cell lines and primary cultures of normal cells including melanocytes reported in Example 1 was confirmed by immunohistochemistry with the tumor lines and cultured normal cells and also with frozen sections of metastatic melanoma  
15 and carcinoma tissues, a benign nevus and three normal tissues including skin: V86 reacted specifically with melanoma cells in the melanoma tissue and cell lines and did not react with any of the normal cells or other tumor cells or the benign nevus. V86 appears to bind to  
20 a cell surface antigen expressed by all of the metastatic melanomas tested and not by normal cells or other tumors.

Although V86 was isolated from a fusion phage library designed to display the antibody repertoire of a  
25 cancer patient as scFv molecules and therefore should have contained both a V $\mu$  and V $\kappa$  domain, most of the V $\kappa$  domain is missing because an extraneous cloning site located near the 5'-end of the V $\kappa$  cDNA was cleaved during construction of the library, resulting in a deletion of  
30 the distal V $\kappa$  region. Despite the absence of a V $\kappa$  partner V86, appears to be one of the most specific anti-melanoma antibodies isolated from the library. It was shown in other studies that an isolated heavy chain of a monoclonal antibody can retain the specificity of the intact  
35 antibody for its cognate determinant, although the affin-

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ity is usually reduced by one or two orders of magnitude (Haber, E. & Richards, F.F., (1963) *Proc. Royal Soc. Series B* 166, 176-187 and Jaton, J.C., et al. (1968) *Biochem.* 7, 4185-4195). A similar finding was reported for V<sub>H</sub> antibodies synthesized from spleen DNA of mice immunized with lysozyme: The specificity for lysozyme was retained but the affinity was about 10-fold weaker as compared to a complete monoclonal anti-lysozyme antibody Ward, E.S., et al. (1989) *Nature* 341, 544-5467). To determine the effect of linking a V<sub>L</sub> domain to V86 on its specificity and affinity for melanoma cells, two scFv fusion phage libraries was constructed, expressing the V86 domain in random pairwise combinations with either V<sub>λ</sub> or V<sub>κ</sub> domains derived from the original scFv library used for the isolation of V86. All of the different V<sub>λ</sub> domains tested as partners for V86 prevented binding to melanoma cells; binding occurred with most of the different V<sub>κ</sub> domains tested but it was generally weaker than the binding of V86. Thus the V86 single-domain V<sub>H</sub> antibody binds more strongly to melanoma cells than do most of the V86 scFv antibodies containing randomly paired V<sub>L</sub> domains. Although the affinity of V86 for melanoma cells is usually reduced by addition of a V<sub>κ</sub> domain, the strict specificity for melanoma cells is not affected, consistent with other evidence that the V<sub>H</sub> domain alone can determine antibody specificity.

The anti-tumor antibodies expressed by the mature B cells in the PBL of vaccinated cancer patients, such as the patient from whom V86 was derived, have probably been subjected to affinity maturation which optimizes the specificity and affinity of an antibody for its cognate determinant. However, because construction of a scFv or Fab library involves random pairings between highly complex populations of V<sub>H</sub> and V<sub>L</sub> cDNAs, there is virtually no chance that the original combination of V<sub>H</sub> and V<sub>L</sub> partners for any antibody will be included in a library of the

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usual size. Pairing a  $V_H$  domain with a new  $V_L$  partner usually results in a less active or inactive antibody, as shown here for V86. Consequently  $V_H$  domains that could function as anti-tumor  $V_H$  antibodies might remain undetected in single scFv or Fab libraries because of an incompatible  $V_L$  partner. To test whether random combinatorial antibody libraries might be circumvented by using  $V_H$  libraries to pan for anti-tumor antibodies, a  $V_H$  fusion phage library containing the same population of  $V_H$  genes as the scFv library used for the isolation of V86 was investigated as described in Example 3, which illustrates that additional melanoma-specific antibodies can be cloned from the  $V_H$  library.

A natural repertoire of functional heavy chain antibodies has been reported in the camel (Hammers-Caserman, C., et al. (1993) Nature 363, 446-448). The antibodies are composed of two identical disulfide-linked heavy chains each containing a  $V_H$ ,  $C_H2$  and  $C_H3$  domain. The camel also has a minor population of antibodies with the expected composition of two complete heavy chains and two complete light chains. This remarkable discovery raises intriguing questions concerning the evolutionary and functional significance of heavy chain antibodies. It also provides additional evidence that a functional antibody need not contain a  $V_L$  domain.

### Example 3

In most mammalian antibodies the variable domain of the heavy chain ( $V_H$ ) and the light chain ( $V_L$ ) interact to form the combining site for the cognate epitope. A notable exception is the antibody repertoire of the camel, which consists mostly of molecules containing two heavy chains but no light chains. The heavy chain camelid antibodies react with a broad spectrum of antigens in

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a trypanosome lysate, indicating that the immune system of the camel can generate a diverse repertoire of functional antibodies devoid of a light chain. Earlier studies with isolated heavy and light chains of several mouse monoclonal antibodies had shown that the binding specificity resided entirely in the heavy chain, the light chain contributing only to the affinity. With the introduction of PCR technology for synthesizing the variable domains of antibodies, the specificity and affinity of  $V_H$  and  $V_L$  domains either alone or coupled in an Fab or scFv molecule could be analyzed. Several such studies have demonstrated the dominant role of  $V_H$  domains in determining antibody specificity, and in some cases the affinity of the  $V_H$  domain alone was comparable to the affinity of the complete antibody.

The cloning of the melanoma-specific antibody V86 from a scFv fusion phage library of a melanoma patient vaccinated with autologous tumor cells that were transfected with interferon-gamma gene was described above. Although V86 was isolated from a scFv library it is essentially a  $V_H$  antibody because an extraneous cloning site located near the 5'-end of the  $V_L$  cDNA was cleaved during construction of the library, resulting in a deletion of the distal  $V_L$  region. The ability of V86 to function as a scFv antibody was tested by randomly conjugating to V86  $V_L$  domains from the antibody repertoire of the same patient. All of the  $V_L$  domains tested as partners for V86 either reduced or eliminated its affinity for melanoma cells, indicating that V86 functions better as a  $V_H$  than as a scFv anti-melanoma antibody. This result suggests that  $V_H$  libraries could provide access to tumor-specific antibodies that might not be detected in scFv or Fab combinatorial libraries because of the incompatibility of most randomly paired  $V_H$  and  $V_L$  domains. In this Example, a  $V_H$  and a scFv fusion phage library constructed from the antibody repertoire of a vaccinated melanoma patient as a

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source of melanoma-specific antibodies are compared. The results of this comparison demonstrate that melanoma-specific antibodies containing  $V_H$  domains with distant CDR regions can be cloned from the two libraries.

5           A  $V_H$  and a scFv fusion phage library was constructed from the antibody repertoire of the vaccinated melanoma patient DM414 described above. Poly (A)<sup>+</sup> RNA was isolated from the PBL samples, and first-strand cDNAs were synthesized with random hexamers and oligo dT primers, using kits purchased from Invitrogen and BRL. The coding regions for the  $V_H$ - $C_H1$  heavy chains were amplified by PCR using the primers below:

          A. Constant region forward-primer of IgG:  
GTC CAC CTT GGT GTT GCT GGG CTT (SEQ ID NO. 41)

15           b.  $V_H$  back-primers with *Sfi*I site:

$V_H$  1,4,6:   ATG GCT CAG GGT TCG GCC GAC GTG GCC CAG GTR  
                  CAG CTG SWG SAG TCK GG (SEQ ID NO. 42)

$V_H$  2:       ATG GCT CAG GGT TCG GCC GAC GTG GCC CAG GTC  
                  AAC TTA AGG GAG TCT GG (SEQ ID NO. 43)

20    $V_H$  3,5:   ATG GCT CAG GGT TCG GCC GAC GTG GCC GAG GTG  
                  CAG CTG KTG SAG TCT GS (SEQ ID NO. 44)

Each PCR mixture containing 2  $\mu$ l from the first-strand cDNA synthesis, 50 pM of a constant-region forward-primer, 50 pM of a back-primer, 250  $\mu$ M dNTPs, and 2.5 units of  
25   Taq polymerase in buffer as provided (Boehringer Mannheim).

          A "touchdown" PCR protocol was used as follows: three cycles each of denaturation at 94°C for 1 minute, annealing for 2 minutes, and elongation at 74°C for 3  
30   minutes; the annealing temperature was varied from 55°C to 40°C in steps of 1°C. The "touchdown" cycles were followed with 10 cycles of annealing to a temperature of 45°C and a 10-minute extension at 74°C. The PCR products were purified by electrophoresis in 1% agarose gel and

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extraction from the gel using Qiaex Kit C (Qiagen); the purified DNAs were dissolved in 40  $\mu$ l of TZ buffer.

For the next PCR step, the joining-region heavy chain ( $J_H$ ) forward primer with *Sfi*I site, ACG TTC GGC CCC AGC GGC CCC GCT ACC CCC GCC TCC TGA RGA GAC GGT GAC CRK KGT YCC (SEQ ID NO. 45) were used in combinations with the  $V_H$  back-primers listed above. The PCR reagents and conditions were the same as above, except that the primer concentrations were 10 pM and the reaction involved 30 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute followed by extension at 72°C for 10 minutes. The PCR products were purified as above.

The  $V_H$  cDNAs and the replicative form of DNA of the *fUSE5* vector were digested with *Sfi*I and purified by electrophoresis in 1% low-melting-temperature agarose gel. Ligation of the cDNA with the vector DNA was done in 100  $\mu$ l of reaction mixture containing 0.8  $\mu$ g of vector DNA, and 2000 units of  $T_4$  DNA ligase for 16 hours at 16°C. The ligation products were purified by extraction with phenol/chloroform and precipitation with ethanol and were dissolved in 20  $\mu$ l of water. The entire purified DNA sample was used to transform DH10B ElectroMax cells (Gibco/BRL), and the cells were plated on 2xTY agar medium and tetracycline at 12.5  $\mu$ g/ml in 24 x 42 cm trays. The size of the library constructed from the PBL of patient DM414 in the vaccination trial at Duke University Medical Center was about  $3 \times 10^8$  independent clones.

Melanoma-specific  $V_H$  and scFv antibodies containing  $V_H$  domains with distinct CDR3 regions were cloned from the two libraries. The specificities of the antibodies were tested by ELISA and immunohistochemistry with melanoma and other tumor cell lines and primary cultures of three normal cell types, and with frozen sections of metastatic melanoma and normal skin. The antibodies bound only to

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the melanoma cells in all tests. Thus, both  $V_R$  and scFv fusion phage libraries can yield tumor-specific antibodies. Sequence results are set out in SEQ ID NOs 46 to 51.

5           The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon  
10       reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention as defined in the appended claims. The claims are meant to cover the claimed components and steps in any sequence which is  
15       effective to meet the objectives there intended, unless the context specifically indicates the contrary.

The references cited above are hereby incorporated herein in their entireties by reference.

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SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANTS: Alan Garen  
Xiaohong Cai
- (ii) TITLE OF INVENTION: Human Anti-Tumor Monoclonal Antibodies
- (iii) NUMBER OF SEQUENCES: 51
- (iv) CORRESPONDENCE ADDRESS
  - (A) ADDRESSEE: Department of Molecular Biophysics  
and Biochemistry, Yale University
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  - (C) CITY: New Haven
  - (D) STATE: Connecticut
  - (E) COUNTRY: United States of America
  - (F) ZIP CODE: 06520-8114
- (v) COMPUTER READABLE FORM
  - (A) MEDIUM TYPE: 3.5" 1.44 Mb diskette
  - (B) COMPUTER: IBM PC
  - (C) OPERATING SYSTEM: MS DOS
  - (D) SOFTWARE: Word Processing
- (vi) CURRENT APPLICATION DATA
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: June 28, 1996
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA
  - (A) APPLICATION NUMBER: U.S. 08/497,647
  - (B) FILING DATE: JUNE 30, 1995
  - (C) CLASSIFICATION:
- (viii) ATTORNEY INFORMATION
  - (A) NAME: Mary M. Krinsky
  - (B) REGISTRATION NO.: 32423
  - (C) REFERENCE/DOCKET NUMBER: 1751-P007B.PCT
- (ix) TELECOMMUNICATION INFORMATION
  - (A) TELEPHONE NUMBER: 203-324-6155
  - (B) TELEFAX NUMBER: 203-327-1096

- 43 -

## (2) INFORMATION FOR SEQ ID NO: 1

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

(A) DESCRIPTION: primer used in constructs

## (x) PUBLICATION INFORMATION

(A) AUTHORS: Marks, J.D., et al.

(B) TITLE: "By-passing Immunization"

(C) JOURNAL: *J. Mol. Biol.*

(D) VOLUME: 222

(E) PAGES: 581-597; primer on page 584

(denoted HuIgG1-4CH1FOR)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 1:

GTCCACCTTG GTGTTGCTGG GCTT

24

## (3) INFORMATION FOR SEQ ID NO: 2

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

(A) DESCRIPTION: primer used in constructs

## (x) PUBLICATION INFORMATION

(A) AUTHORS: Marks, J.D., et al.

(B) TITLE: "By-passing Immunization"

(C) JOURNAL: *J. Mol. Biol.*

(D) VOLUME: 222

(E) PAGES: 581-597; primer on page 584

(denoted HuIgMFOR)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 2:

TGGAAGAGGC ACGTTCTTTT CTTT

24

## (4) INFORMATION FOR SEQ ID NO: 3

- 44 -

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 24 residues
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
  - (A) DESCRIPTION: primer used in constructs
- (x) PUBLICATION INFORMATION
  - (A) AUTHORS: Marks, J.D., et al.
  - (B) TITLE: "By-passing Immunization"
  - (C) JOURNAL: *J. Mol. Biol.*
  - (D) VOLUME: 222
  - (E) PAGES: 581-597; primer on page 584  
(denoted HuGκFOR)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 3:  
AGACTCTCCC CTGTTGAAGC TCTT

24

## (5) INFORMATION FOR SEQ ID NO: 4

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 27 residues
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
  - (A) DESCRIPTION: primer used in constructs
- (x) PUBLICATION INFORMATION
  - (A) AUTHORS: Marks, J.D., et al.
  - (B) TITLE: "By-passing Immunization"
  - (C) JOURNAL: *J. Mol. Biol.*
  - (D) VOLUME: 222
  - (E) PAGES: 581-597; primer on page 584  
(denoted HuGλFOR)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 4:  
TGAAGATTCT GTAGGGGCCA CTGTCTT

27

## (6) INFORMATION FOR SEQ ID NO: 5

- (i) SEQUENCE CHARACTERISTICS

- 45 -

- (A) LENGTH: 50 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (A) DESCRIPTION: primer used in constructs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 5:

ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG GTRCAGCTGS WGSAGTCKGG

50

(7) INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 50 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (A) DESCRIPTION: primer used in constructs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 6:

ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG GTCAACTTAA GGGAGTCTGG

50

(8) INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 50 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- (A) DESCRIPTION: primer used in constructs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 7:

ATGGCTCAGG GTTCGGCCGA CGTGGCCGAG GTGCAGCTGK TGSAGTCTGS

50

(9) INFORMATION FOR SEQ ID NO: 8

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 59 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- 46 -

- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 8:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCTGACA TCSWGATGAC 50  
CCAGTCTCC 59
- (10) INFORMATION FOR SEQ ID NO: 9
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 59 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 9:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCAGAWR TTGTGMTGAC 50  
KCACTCTCC 59
- (11) INFORMATION FOR SEQ ID NO: 10
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 59 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 10:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCAGAAA CGACACTCAC 50  
GCAGTCTCC 59
- (12) INFORMATION FOR SEQ ID NO: 11
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 59 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- 47 -

- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 11:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCACAGT CTGYSYTGAC 50  
KCAGCCGCC 59
- (13) INFORMATION FOR SEQ ID NO: 12
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 59 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 12:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCATCYT MTGWGCTGAC 50  
TCAGSMACC 59
- (14) INFORMATION FOR SEQ ID NO: 13
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 59 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 13:  
GGCTCGGGCG GTGGTGGGTC GGGTGGCGGC GGATCACASG YTRTACTGAC 50  
TCAACCGYC 59
- (15) INFORMATION FOR SEQ ID NO: 14
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 60 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA

- 48 -

- (A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 14:
- GCCACCCGAC CCACCACCGC CCGAGCCACC GCCACCTGAR GAGACGGTGA 50  
CCRKKGTGCC 60
- (16) INFORMATION FOR SEQ ID NO: 15
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 51 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 15:
- AGTCTAACGT TCGGCCCCAG CGGCCCCACG TTTGATYTCC ASCTTGGTCC C 51
- (17) INFORMATION FOR SEQ ID NO: 16
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 51 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 16:
- AGTCTAACGT TCGGCCCCAG CGGCCCCACG TTTKATMTCC ASYYKKGTTCC C 51
- (18) INFORMATION FOR SEQ ID NO: 17
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 51 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (A) DESCRIPTION: primer used in constructs
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 17:
- AGTCTAACGT TCGGCCCCAG CGGCCCCACC TARRACGGTS ASCTKGGTCC C 51

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## (19) INFORMATION FOR SEQ ID NO: 18

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 23 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

- (A) DESCRIPTION: primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 18:

ATTATTATTC GCAATTCCTT TAG

23

## (20) INFORMATION FOR SEQ ID NO: 19

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 24 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

- (A) DESCRIPTION: primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 19:

GAATTTTCTG TATGAGGTTT TGCT

24

## (21) INFORMATION FOR SEQ ID NO: 20

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 121 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE:

- (A) DESCRIPTION: polypeptide

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

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(D) CLONE: H18

(ix) FEATURE

(A) NAME: heavy chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 20:

Glu	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Glu	Val	Lys	Lys	Pro	Gly		5	10	15
Ala	Ser	Val	Lys	Ile	Ser	Cys	Lys	Val	Ser	Gly	His	Pro	Phe	Thr		20	25	30
Asn	His	Trp	Phe	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu		35	40	45
Glu	Trp	Met	Gly	Trp	Val	Ser	Pro	Asn	Thr	Gly	Ala	Thr	Lys	Tyr		50	55	60
Ala	Glu	Asn	Phe	Gln	Gly	Arg	Val	Thr	Asn	Thr	Trp	Asp	Thr	Ser		65	70	75
Ile	Leu	Thr	Ala	Tyr	Met	Glu	Leu	Arg	Ser	Leu	Thr	Ser	Glu	Asp		80	85	90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Ala	Ala	Ala	Gly	Ser	Ser	Tyr	Tyr		95	100	105
Phe	Gly	Met	Asp	Ile	Trp	Ala	Lys	Gly	Ile	Pro	Val	Thr	Val	Ser		110	115	120

Ser

(22) INFORMATION FOR SEQ ID NO: 21

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 111 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

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(D) CLONE: H18

(ix) FEATURE

(A) NAME: light chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 21:

```

Ser Tyr Glu Leu Thr Gln Gln Pro Ser Ala Ser Gly Thr Pro Gly
              5              10              15
Gln Arg Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly
              20              25              30
Ser Asn Tyr Val Tyr Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro
              35              40              45
Lys Leu Leu Ile Tyr Arg Asn Asn Gln Arg Pro Ser Gly Val Pro
              50              55              60
Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu Ala
              65              70              75
Ile Ser Gly Leu Arg Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gln
              80              85              90
Ser Tyr Asp Asn Ser Leu Asn Gly Tyr Val Phe Gly Gly Gly Thr
              95              100             105
Gln Leu Thr Val Leu Gly
              110

```

(23) INFORMATION FOR SEQ ID NO: 22

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 119 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct

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(D) CLONE: G57

(ix) FEATURE

(A) NAME: heavy chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 22:

```

Gln Val Asn Leu Arg Glu Ser Gly Gly Gly Leu Val Lys Pro Gly
      5                      10                      15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
      20                      25                      30
Asp Tyr Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu
      35                      40                      45
Glu Trp Val Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr
      50                      55                      60
Thr Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
      65                      70                      75
Lys Asn Thr Leu Tyr Leu Gln Asn Ser Leu Arg Ala Glu Asp Thr
      80                      85                      90
Ala Ile Tyr Tyr Cys Ala Lys Glu Met Asn Tyr Phe Ser His Ala
      95                      100                     105
Met Asp Val Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
      110                     115

```

(24) INFORMATION FOR SEQ ID NO: 23

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 111 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct

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(D) CLONE: G57

(ix) FEATURE

(A) NAME: light chain

(D) OTHER INFORMATION: Xaa in position 6 is His or Gln

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 23:

Gln Ser Val Leu Thr Xaa Pro Pro Ser Ala Ser Gly Thr Pro Gly  
                                   5                                  10                                  15

Gln Arg Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly  
                                   20                                  25                                  30

Ser Asn Tyr Val Tyr Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro  
                                   35                                  40                                  45

Lys Leu Leu Ile Tyr Arg Asn Asn Gln Arg Pro Ser Gly Val Pro  
                                   50                                  55                                  60

Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu Ala  
                                   65                                  70                                  75

Ile Ser Gly Leu Arg Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Ala  
                                   80                                  85                                  90

Ser Trp Asp Asp Ser Leu Arg Gly Trp Val Phe Gly Gly Gly Thr  
                                   95                                  100                                  105

Gln Leu Thr Val Leu Ser  
                                   110

(25) INFORMATION FOR SEQ ID NO: 24

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 118 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

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## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from  
fUSE5 fusion phage construct

(D) CLONE: D33

## (ix) FEATURE

(A) NAME: heavy chain

(D) OTHER INFORMATION: Xaa in position 81 is Leu or  
Thr

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 24:

Glu	Val	Gln	Leu	Met	Glu	Ser	Gly	Ala	Glu	Val	Lys	Lys	Pro	Gly	
				5					10					15	
Ala	Ser	Val	Lys	Ile	Ser	Cys	Cys	Ser	Gly	His	Pro	Phe	Thr	Asn	
				20					25					30	
His	Trp	Phe	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	
				35					40					45	
Trp	Met	Gly	Trp	Val	Ser	Pro	Asn	Thr	Gly	Ala	Thr	Lys	Thr	Ala	
				50					55					60	
Glu	Met	Phe	Gln	Gly	Arg	Val	Thr	Asn	Thr	Trp	Asp	Thr	Ser	Ile	
				65					70					75	
Xaa	Thr	Ala	Tyr	Met	Gly	Leu	Arg	Ser	Leu	Thr	Ser	Glu	Asp	Thr	
				80					85					90	
Pro	Val	Tyr	Tyr	Cys	Ala	Thr	Ala	Ala	Gly	Ser	Ser	Tyr	Tyr	Phe	
				95					100					105	
Gly	Asn	Asp	Ile	Trp	Arg	Lys	Gly	Ile	Pro	Val	Thr	Val			
				110					115						

## (26) INFORMATION FOR SEQ ID NO: 25

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 113 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

## (vi) ORIGINAL SOURCE:

- 55 -

- (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct
- (D) CLONE: D33
- (ix) FEATURE
- (A) NAME: light chain
- (D) OTHER INFORMATION: Xaa at position 47 is His or Gly and Xaa at position 95 is His or Gln
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 25:

```

Glu Ile Leu Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro
      5                      10                      15
Gly Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Thr Leu Val
      20                      25                      30
Phe Ser Asp Gly His Asn Tyr Val Asn Trp Phe Gln Gln Arg Pro
      35                      40                      45
Ala Xaa Ser Pro Arg Arg Leu Ile Tyr Glu Leu Ser Asn Arg Asp
      50                      55                      60
Pro Gly Val Pro Asp Arg Phe Ser Asp Ser Gly Ser Asp Thr Asp
      65                      70                      75
Leu Thr Leu Lys Ile Ser Arg Val Gln Ala Glu Asp Val Gly Val
      80                      85                      90
Tyr Tyr Cys Ile Xaa Gly Thr Leu Cys Pro Phe Thr Phe Gly Gly
      95                      100                     105
Gly Thr Arg Val Glu Ile Lys Arg
      110

```

## (27) INFORMATION FOR SEQ ID NO: 26

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 126 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:

- 56 -

- (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct
- (D) CLONE: Z78
- (ix) FEATURE
- (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 26:

Gln	Val	Asn	Leu	Arg	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Trp	5	10	15
Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ser	Ala	Ser	Gly	Phe	Thr	Phe	Ser	20	25	30
Gly	His	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	35	40	45
Glu	Ser	Val	Ser	Ala	Ile	Ser	Gly	Asn	Gly	Gly	Ser	Thr	Tyr	Tyr	50	55	60
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser	65	70	75
Leu	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Ser	Ser	Leu	Arg	Ala	Glu	Asp	80	85	90
Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Arg	Asp	Trp	Tyr	Pro	Asp	Ser	Trp	95	100	105
Ser	Gly	Tyr	Ala	Val	Asp	Gly	Leu	Asp	Val	Trp	Ala	Lys	Gly	Thr	110	115	120
Thr	Val	Thr	Val	Ser	Ser										125		

## (28) INFORMATION FOR SEQ ID NO: 27

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 111 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

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- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
- (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: DM414 scFv antibodies obtained from *FUSE5* fusion phage construct
- (D) CLONE: Z78
- (ix) FEATURE
- (A) NAME: light chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 27:

Gln	Ser	Ala	Leu	Thr	Gln	Pro	Pro	Ser	Val	Ser	Glu	Ala	Pro	Arg	5	10	15
Gln	Arg	Val	Thr	Ile	Ser	Cys	Ser	Gly	Ser	Ser	Ser	Asn	Ile	Gly	20	25	30
Asn	Asn	Ala	Val	Asn	Trp	Tyr	Gln	Gln	Leu	Pro	Gly	Lys	Ala	Pro	35	40	45
Lys	Leu	Leu	Ile	Tyr	Tyr	Asp	Asp	Leu	Leu	Pro	Ser	Gly	Val	Ser	50	55	60
<hr/>																	
Asp	Arg	Phe	Ser	Gly	Ser	Lys	Ser	Gly	Thr	Ser	Ala	Ser	Leu	Ala	65	70	75
Ile	Ser	Gly	Leu	Gln	Ser	Glu	Asp	Glu	Ala	Asp	Tyr	Tyr	Cys	Ala	80	85	90
Ala	Trp	Asp	Asp	Ser	Leu	Asn	Gly	Pro	Val	Phe	Gly	Gly	Gly	Thr	95	100	105
Lys	Leu	Thr	Val	Leu	Gly										115		

## (29) INFORMATION FOR SEQ ID NO: 28

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 131 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

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- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
- (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct
- (D) CLONE: V13
- (ix) FEATURE
- (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 28:

Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	5	10	15
Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	20	25	30
Ser	Tyr	Ala	Met	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	35	40	45
Glu	Trp	Val	Ser	Ala	Ile	Ser	Gly	Ser	Gly	Gly	Ser	Thr	Tyr	Tyr	50	55	60
<hr/>																	
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser	65	70	75
Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	80	85	90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Lys	Gly	Val	Ala	Pro	Phe	Asp	Tyr	95	100	105
Trp	Gly	Gln	Gly	Thr	Pro	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	110	115	120
Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser					125	130	

## (30) INFORMATION FOR SEQ ID NO: 29

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 108 residues

- 59 -

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct
  - (D) CLONE: V13
- (ix) FEATURE
  - (A) NAME: light chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 29:

```

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
      5                                10                        15
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
      20                                25                        30
Ser Tyr Leu Asn Tyr Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
      35                                40                        45
Leu Leu Ile Tyr Lys Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
      50                                55                        60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile
      65                                70                        75
Ser Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
      80                                85                        90
Tyr Asn Ser Tyr Ser Arg Thr Phe Gly Gln Gly Thr Lys Leu Glu
      95                                100                       105
Ile Lys Arg

```

## (31) INFORMATION FOR SEQ ID NO: 30

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 122 residues
  - (B) TYPE: amino acid

- 60 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: DM414 scFv antibodies obtained from FUSE5 fusion phage construct
  - (D) CLONE: V73
- (ix) FEATURE
  - (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 30:

```

Gln Val Gln Leu Val Gln Ser Gly Ser Arg Gly Val Lys Xaa Arg
      5                      10                      15
Gly Val Ser Xaa Ile Ser Cys Lys Gly Ser Gly Tyr Thr Phe Thr
      20                      25                      30
Ser Tyr Trp Ile Gly Trp Val Arg Gln Ile Pro Gly Lys Gly Leu
      35                      40                      45
Glu Trp Met Gly Ile Ile Tyr Pro Gly Asp Ser Asp Thr Arg Tyr
      50                      55                      60
Arg Pro Ser Phe Gln Gly Gln Val Thr Ile Ser Ala Asp Lys Ser
      65                      70                      75
Ile Ser Thr Ala Tyr Leu Gln Trp Ser Ser Leu Lys Ala Ser Asp
      80                      85                      90
Thr Ala Met Tyr Tyr Cys Ala Arg Leu Thr Val Asp Asp Tyr Gly
      95                      100                     105
Gly Asn Thr Pro Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val
      110                     115                     120
Ser Ser

```

## (32) INFORMATION FOR SEQ ID NO: 31

## (i) SEQUENCE CHARACTERISTICS

- 61 -

- (A) LENGTH: 112 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct
  - (D) CLONE: V73
- (ix) FEATURE
  - (A) NAME: light chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 31:

```

Gln Ser Ala Leu Thr Gln Pro Pro Ser Val Ser Ala Ala Pro Gly
      5                      10                      15
Gln Lys Val Thr Xaa Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile
      20                      25                      30
Gly Asn Asn Tyr Val Ser Trp Tyr Gln Gln Leu Pro Gly Thr Ala
      35                      40                      45
Pro Lys Leu Leu Ile Tyr Glu Asn Asn Lys Arg Pro Ser Gly Ile
      50                      55                      60
Pro Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Thr Leu
      65                      70                      75
Gly Ile Thr Gly Leu Gln Thr Gly Asp Glu Ala Asp Tyr Tyr Cys
      80                      85                      90
Gly Thr Trp Asp Ser Ser Leu Ser Ala Glu Val Phe Gly Thr Gly
      95                      100                     105
Thr Gln Leu Thr Val Leu Gly
      110

```

## (33) INFORMATION FOR SEQ ID NO: 32

- (i) SEQUENCE CHARACTERISTICS

- 62 -

- (A) LENGTH: 140 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct
  - (D) CLONE: V86
- (ix) FEATURE
  - (A) NAME: heavy chain and linker
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 32:

```

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Gln Pro Gly
      5                               10                      15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
      20                               25                      30
Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
      35                               40                      45
Glu Trp Val Ala Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr
      50                               55                      60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
      65                               70                      75
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      80                               85                      90
Thr Ala Val Tyr Tyr Cys Ala Arg Gly Trp Gly Leu Arg Gly Glu
      95                               100                     105
Glu Gly Asp Tyr Tyr Met Asp Val Trp Gly Lys Gly Thr Met Val
      110                              115                     120
Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
      125                              130                     135
Gly Gly Gly Ser Ser
      140

```

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## (34) INFORMATION FOR SEQ ID NO: 33

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 24 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct
  - (D) CLONE: V86
- (ix) FEATURE
  - (A) NAME: light chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 33:

Ser Tyr Glu Leu Thr Gln Glu Pro Arg Gly Gly Gly Thr Gln Leu  
                                  5                                  10                                  15  
Thr Val Leu Gly Gly Ala Ala Gly Ala  
                                  20

## (35) INFORMATION FOR SEQ ID NO: 34

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 119 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

- 64 -

- (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: DM414 scFv antibodies obtained from fUSE5 fusion phage construct
- (D) CLONE: V373
- (ix) FEATURE
- (A) NAME: heavy chain
- (D) OTHER INFORMATION: Xaa at position 3 is Gln or Xaa and Xaa at position 19 is Xaa or Ser
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 34:

```

Glu Val Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
      5                                10                                15
Xaa Xaa Xaa Xaa Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Ser
      20                                25                                30
Tyr Ile His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp
      35                                40                                45
Met Gly Val Ile Asn Pro Ser Gly Gly Asn Thr Ile Tyr Ala Arg
      50                                55                                60
Asn Phe Gln Gly Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser
      65                                70                                75
Thr Val Tyr Met Glu Leu Ser Ser Leu Arg Phe Glu Asp Thr Ala
      80                                85                                90
Val Tyr Tyr Cys Ala Arg Asp Arg Arg Tyr Cys Ser Gly Gly Ser
      95                                100                               105
Cys Tyr Ala Glu Val Val Tyr Trp Gly Gln Gly Thr Thr Val
      110                               115

```

(36) INFORMATION FOR SEQ ID NO: 35

- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 111 residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
- (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:

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(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

(D) CLONE: V373

(ix) FEATURE

(A) NAME: light chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 35:

```

Gln Ser Ala Leu Thr Gln Pro Pro Ser Val Ser Ala Ala Pro Gly
      5                               10                15

Gln Lys Val Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly
      20                               25                30

Asn Asn Tyr Val Ser Trp Tyr Gln Arg Leu Pro Gly Thr Ala Pro
      35                               40                45

Lys Leu Leu Ile Tyr Glu Asn Asn Lys Arg Pro Ser Gly Ile Pro
      50                               55                60

Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Thr Leu Ala
      65                               70                75

Ile Thr Gly Leu Gln Ala Glu Asp Glu Ala Gly Tyr Tyr Cys Gln
      80                               85                90

Ser Tyr Asp Ser Ser Leu Ser Gly Tyr Val Phe Gly Thr Gly Thr
      95                               100               105

Lys Leu Thr Val Leu Gly
      110

```

(37) INFORMATION FOR SEQ ID NO: 36

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 116 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

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(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

(ix) FEATURE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 36:

Glu	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly
				5					10					15
Gly	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser
				20					25					30
Ser	Tyr	Thr	Met	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
				35					40					45
Glu	Trp	Val	Ser	Ala	Ile	Ser	Gly	Ser	Gly	Gly	Ser	Thr	Tyr	Tyr
				50					55					60
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser
				65					70					75
Lys	Asn	Thr	Arg	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp
				80					85					90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Lys	Gly	Val	Ala	Pro	Phe	Asp	Tyr
				95					100					105
Trp	Gly	Glu	Gly	Thr	Pro	Val	Thr	Val	Ser	Ser				
				110					115					

(38) INFORMATION FOR SEQ ID NO: 37

### (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 108 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

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(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

(D) CLONE: V474

(ix) FEATURE

(A) NAME: light chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 37:

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	5	10	15
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Ser	Ile	Ser	20	25	30
Ser	Tyr	Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Glu	Gly	Lys	Ala	Pro	Lys	35	40	45
Leu	Leu	Ile	Tyr	Lys	Ala	Ser	Ser	Leu	Glu	Ser	Gly	Val	Pro	Ser	50	55	60
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr	Leu	Thr	Ile	65	70	75
Ser	Ser	Leu	Gln	Pro	Asp	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	80	85	90
Tyr	Asn	Ser	Ser	Ser	Arg	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Leu	Gly	95	100	105
Ile	Lys	Arg															

(39) INFORMATION FOR SEQ ID NO: 38

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 123 residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: polypeptide

(vi) ORIGINAL SOURCE:

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(A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)

(B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: DM414 scFv antibodies obtained from *fUSE5* fusion phage construct

(D) CLONE: V575

(ix) FEATURE

(A) NAME: heavy chain

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 38:

Gln	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Val	Val	Gln	Pro	Gly			
				5					10					15			
Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser			
				20					25					30			
Ser	Tyr	Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu			
				35					40					45			
Glu	Trp	Val	Ala	Val	Ile	Ser	Tyr	Asp	Gly	Ser	Asn	Lys	Tyr	Tyr			
				50					55					60			
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser			
				65					70					75			
Lys	Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu			
				80					85					90			
Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Gly	Phe	Pro	Tyr	Gly	Gly			
				95					100					105			
Asn	Ser	Asp	Tyr	Gly	Met	Asp	Val	Trp	Asp	His	Gly	Thr	Gln	Val			
				110					115					120			
Thr	Val	Ser															

(40) INFORMATION FOR SEQ ID NO: 39

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 17 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO 39:  
TGATTTTCTG TATGAGG 17
- (41) INFORMATION FOR SEQ ID NO: 40  
(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 21 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO 40:  
ACCCGACCCA CCACCGCCCG A 21
- (42) INFORMATION FOR SEQ ID NO: 41  
(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 24 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO 41:  
GTCCACCTTG GTGTTGCTGG GCTT 24
- (43) INFORMATION FOR SEQ ID NO: 42  
(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 50 residues  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA  
(A) DESCRIPTION: primer used in constructs  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO 42:  
ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG GTRCAGCTGS WGSAGTCKGG 50

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## (44) INFORMATION FOR SEQ ID NO: 43

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 50 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

(A) DESCRIPTION: primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 43:

ATGGCTCAGG GTTCGGCCGA CGTGGCCCAG GTCAACTTAA GGGAGTCTGG 50

## (45) INFORMATION FOR SEQ ID NO: 44

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 50 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

(A) DESCRIPTION: primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 44:

ATGGCTCAGG GTTCGGCCGA CGTGGCCGAG GTGCAGCTGK TGSAGTCTGS 50

## (46) INFORMATION FOR SEQ ID NO: 45

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 60 residues

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

(A) DESCRIPTION: primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 45:

ACGTTTCGGCC CCAGCGGCCC CGCTACCCCC GCCTCCTGAR GAGACGGTGA 50

CCRKKGTYCC 60

## (47) INFORMATION FOR SEQ ID NO: 46

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 117 residues

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- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from *FUSE5* fusion phage construct
  - (D) CLONE: C55
- (ix) FEATURE
  - (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 46:

```

Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Gln Pro Gly
      5                                10                        15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
      20                                25                        30
Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
      35                                40                        45
Glu Trp Val Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr
      50                                55                        60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
      65                                70                        75
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      80                                85                        90
Ala Ala Val Tyr Tyr Cys Ala Thr Gly Gly Gly Leu Leu Ser Asp
      95                                100                       105
Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
      110                               115

```

(48) INFORMATION FOR SEQ ID NO: 47

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- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 124 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from *FUSE5* fusion phage construct
  - (D) CLONE: E26
- (ix) FEATURE
  - (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 47:

```

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Gln Pro Gly
      5                                10                                15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Tyr
      20                                25                                30
Ser Tyr Ala Met Ser Trp Val Arg Gln Thr Pro Gly Lys Gly Leu
      35                                40                                45
Glu Trp Val Ser Ala Ile Gly Gly Ser Gly Val Ser Thr Tyr Tyr
      50                                55                                60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
      65                                70                                75
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      80                                85                                90
Thr Ala Val Tyr Tyr Cys Ala Arg Gly Trp Gly Leu Arg Gly Glu
      95                                100                               105
Glu Gly Asp Tyr Tyr Met Asp Val Trp Gly Lys Gly Thr Gln Val
      110                               115                               120
Thr Val Ser Ser

```

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## (49) INFORMATION FOR SEQ ID NO: 48

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 126 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from fUSES fusion phage construct
  - (D) CLONE: F2
- (ix) FEATURE
  - (A) NAME: heavy chain

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 48:

Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Gly	Gly	Val	Val	Gln	Pro	Gly	5	10	15
Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	20	25	30
Asp	Cys	Tyr	Met	Ser	Trp	Ile	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	35	40	45
Glu	Trp	Val	Ser	Tyr	Ile	Ser	Ser	Ser	Ser	Ser	Thr	Ile	Tyr	Tyr	50	55	60
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	65	70	75
Lys	Asn	Ser	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Asp	Glu	Asp	80	85	90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Asp	Ser	Ser	Gly	Ser	Tyr	Ser	95	100	105

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Glu Arg Asp Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly Thr  
                           110                          115                          120  
 Pro Val Thr Val Ser Ser  
                           125

## (50) INFORMATION FOR SEQ ID NO: 49

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 112 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from *fUSE5* fusion phage construct
  - (D) CLONE: F2
- (ix) FEATURE
  - (A) NAME: light chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 49:

Gln Ser Ala Leu Thr Glu Pro Pro Ser Val Ser Gly Ala Pro Gly  
                           5                          10                          15  
 Gln Arg Val Thr Ile Ser Cys Thr Gly Ser Thr Ser Asn Ile Gly  
                           20                          25                          30  
 Ala Gly Tyr Asp Val Arg Trp Tyr Gln His Leu Pro Gly Thr Val  
                           35                          40                          45  
 Pro Lys Leu Leu Ile Tyr Thr Asn Ser Ile Arg Pro Ser Gly Val  
                           50                          55                          60  
 Pro Asp Arg Phe Ser Gly Ser Lys Ser Gly Thr Ser Ala Ser Leu  
                           65                          70                          75  
 Thr Ile Asp Gly Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys  
                           80                          85                          90

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Ala Thr Trp Asp Asp Arg Leu Asp Gly Tyr Val Phe Ala Thr Gly  
                             95                            100                            105

Thr Gln Leu Thr Val Leu Gly  
                             110

## (51) INFORMATION FOR SEQ ID NO: 50

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 119 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from fUSES fusion phage construct
  - (D) CLONE: 2-71
- (ix) FEATURE
  - (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 50:

Ala Ala Gly Gly Ala Val Gly Gly Leu Glu Val Lys Lys Pro Gly  
                             5                            10                            15

Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser  
                             20                            25                            30

Ser Tyr Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu  
                             35                            40                            45

Glu Trp Met Gly Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr  
                             50                            55                            60

Ala Gln Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Lys Ser  
                             65                            70                            75

Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp  
                             80                            85                            90

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Thr Ala Val Tyr Tyr Cys Ala Arg Gly Gly Gly Arg Tyr Asp Ala  
                             95                            100                            105  
 Phe Asp Ile Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
                             110                            115

## (52) INFORMATION FOR SEQ ID NO: 51

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 124 residues
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: polypeptide
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Homo sapiens* (melanoma patient immunized with autologous tumor cells)
  - (B) INDIVIDUAL ISOLATE: peripheral blood lymphocytes
- (vii) IMMEDIATE SOURCE:
  - (A) LIBRARY: V<sub>H</sub> antibodies obtained from fUSE5 fusion phage construct
  - (D) CLONE: E-13
- (ix) FEATURE
  - (A) NAME: heavy chain
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 51:

Glu Val Gln Leu Leu Glu Ser Ala Gly Gly Leu Val Gln Pro Gly  
                             5                            10                            15  
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser  
                             20                            25                            30  
 Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu  
                             35                            40                            45  
 Glu Trp Val Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr  
                             50                            55                            60  
 Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser  
                             65                            70                            75  
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp  
                             80                            85                            90

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Thr Ala Val Tyr Tyr Cys Ala Arg Gly Trp Gly Leu Arg Gly Glu  
95 100 105

Glu Gly Asp Tyr Tyr Val Asp Val Trp Gly Lys Gly Thr Lys Val  
110 115 120

Thr Val Leu Gly

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CLAIMS

1. A process for isolating and synthesizing human mono-clonal anti-tumor antibodies comprising:
  - (a) constructing at least one fusion phage li-  
brary from the peripheral blood lymphocytes of a cancer  
5 patient;
  - (b) screening for anti-tumor antibodies in said  
phage library in a binding assay with cultured tumor  
cells of the same type as the patient's tumor;
  - (c) removing extraneous antibodies by absorption  
10 against normal human cells;
  - (d) cloning the phage selected in steps (b) and  
(c);
  - (e) assaying the specificity of said cloned phage  
by incubating the phage with at least two types of cul-  
15 tured normal cells; and
  - (f) further testing the specificity of cloned  
phage that do not bind to either cell line of cultured  
normal cells in further binding assays to cultured tumor  
cells derived from more than one other tumor that is not  
20 the patient's tumor.
2. A process according to claim 1 wherein further test-  
ing of cloned phage comprises binding assays with at  
least five different tumor cell types.
3. A process according to claim 1 wherein the lympho-  
cytes employed in step (a) are derived from cancer pa-  
tients immunized with autologous tumor cells which are  
gene modified to enhance immunogenicity.
4. A process according to claim 3 wherein said gene-  
modified tumor cells are transduced with a cytokine.

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5. A process according to claim 3 wherein the fusion phage library comprises both a single-chain Fv and a V<sub>H</sub> library in an *Escherichia coli* filamentous phage.

6. A process according to claim 2 wherein the binding assays with cultured tumor and normal cells are ELISAs.

7. A process according to claim 2 wherein the specificity of the cloned phage is further tested against sections of primary human tissue.

8. An antibody produced by the process according to claim 1.

9. A process for synthesizing, cloning and selecting human monoclonal single-chain Fv or V<sub>H</sub> anti-melanoma antibodies comprising:

(a) constructing at least one fusion phage library in a filamentous phage vector from the peripheral blood lymphocytes of a cancer patient immunized with autologous melanoma cells that have been gene-modified to enhance the immune response to the melanoma;

(b) screening for anti-melanoma antibodies in said phage library in at least two consecutive binding assays with cultured melanoma cells;

(c) removing extraneous antibodies by absorption against a culture of normal human cells;

(d) cloning the phage that bind to the cultured melanoma cells but not to the normal human cells;

(e) assaying the specificity of said cloned phage by ELISA tests with cultured normal endothelial and fibroblast cells; and

(f) further testing the specificity of cloned phage that do not bind to either cell line of cultured normal cells in a further ELISA test using cultured tumor cells.

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10. A process according to claim 9 wherein the further binding assay of cloned phage to cultured tumor cells in step (f) comprises a series of incubations with at least five different tumor cell cultures comprising autologous melanoma cells, other melanoma cells, melanocytes, and unrelated tumor cells.

11. A process according to claim 10 wherein the cultured tumor cells employed in the further binding assay comprise a glioma line derived from glial cells that share a common lineage with melanocytes, in order to identify melanocytic lineage-specific antibodies.

12. A process according to claim 10 wherein the unrelated tumor cells are selected from the group consisting of pancreatic carcinoma cells, breast carcinoma cells, ovarian carcinoma cells, colorectal carcinoma cells, prostate carcinoma cells, gastric carcinoma cells, renal carcinoma cells, and mixtures thereof.

13. A process according to claim 10 wherein the gene modification of autologous melanoma cells is transduction with a cytokine.

14. A process according to claim 9 wherein the specificity of the cloned phage is further tested against sections of primary human tissue.

15. An antibody produced according to the process of claim 9.

16. An antibody according to claim 15 that is melanoma-specific.

17. An antibody according to claim 16 that is melanocytic lineage-specific.

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18. An antibody according to claim 16 that is specific to melanoma and other tumors.

19. An antibody selected from the group consisting of SEQ ID NOS 20 to 33, residues 20 to 119 of SEQ ID NO 34, SEQ ID NOS 34 to 38, and SEQ ID NOS 46 to 51.

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 6 : C07K 16/00, G01N 33/53, C12N 15/00, C12P 19/34, C07H 21/04, C12Q 1/68, A61K 39/395</p>	<p>A3</p>	<p>(11) International Publication Number: <b>WO 97/02479</b>  (43) International Publication Date: 23 January 1997 (23.01.97)</p>
<p>(21) International Application Number: PCT/IB96/01032  (22) International Filing Date: 28 June 1996 (28.06.96)  (30) Priority Data: 08/497,647 30 June 1995 (30.06.95) US  (71) Applicant (for all designated States except US): YALE UNIVERSITY [US/US]; Yale Station, New Haven, CT 06520 (US).  (72) Inventors; and (75) Inventors/Applicants (for US only): GAREN, Alan [US/US]; 278 Canner Street, New Haven, CT 06511 (US). CAI, Xiaohong [US/US]; 583B Prospect Street, New Haven, CT 06511 (US).  (74) Agent: KRINSKY, Mary, M.; St. Onge Steward Johnston &amp; Reens, 986 Bedford Street, Stamford, CT 06905 (US).</p>		<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  (88) Date of publication of the international search report: 12 June 1997 (12.06.97)</p>
<p>(54) Title: HUMAN MONOCLONAL ANTI-TUMOR ANTIBODIES</p> <p>(57) Abstract</p> <p>Human monoclonal anti-tumor antibodies are isolated from fusion phage single-chain Fv and V<sub>H</sub> antibody libraries constructed from the peripheral blood lymphocytes of immunized cancer patients. Antibodies that bind to tumor cells of the same kind as the patient's are selected, and antibodies that also bind to a human normal cell type are removed. The remaining fusion phage antibodies are cloned and then are tested for binding to at least two normal human cell types. Antibodies that fail to bind to the normal cells are further tested for binding to a panel of tumor cells, typically including the original tumor type and several related and unrelated tumors. Human monoclonal antibodies that bind specifically to the original tumor or also to some other tumors, or that bind to the original tumor and cells from the same developmental lineage, are obtained and sequenced. The selected antibodies can be used to design molecules which are potentially useful for various diagnostic and therapeutic purposes. The single-chain Fv and V<sub>H</sub> libraries from cancer patients are also being used to select antibodies against other targets, such as endothelial cells, which have diagnostic and clinical applications.</p>		

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB96/01032

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.6, 69.7, 71.1, 172.3, 91.2, 252.3; 530/388.4; 435/6; 536/23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
DIALOG (MEDLINE, EMBASE, BIOSIS, WPI)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 90/14430 A1 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 29 November 1990, see entire document.	1-18
Y	MARKS, J.D., et al. By-passing Immunization Human Antibodies from V-gene Libraries Displayed on Phage. J. Mol. Biol. 1991, Vol. 222, pages 581-597, see entire document.	1-18
Y	WILMES, E. et al. Versuche zur Herstellung menschlicher monoklonaler Antikörper gegen Melanome unter Verwendung zervikaler Lymphknoten. Laryng. Rhinol. Otol. 1987, Vol. 66, pages 144-148, see abstract.	9-18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 APRIL 1997

Date of mailing of the international search report

07 MAY 1997

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Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB96/01032

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 19  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
THE CLAIM IS DRAWN TO SPECIFIC SEQ ID NOS BUT THERE IS NO CRF IN THE CASE.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB96/01032

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

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